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(54) Title: METHODS AND COMPOSITIONS FOR MODULATION AND INHIBITION OF TELOMERASE			
(57) Abstract <p>It was found that normal human stem cells produce a regulated non-processive telomerase activity, while cancer cells produce a processive telomerase activity. Nucleotide analogs, such as 7-deaza-2'-deoxyquanosine-5'-triphosphate (7-deaza-dGTP) were found to be substrates for processive telomerase and incorporated into telomeric sequence. The incorporation of this nucleotide subsequently affected the processivity of telomerase, converting processive telomerase to non-processive telomerase. The incorporation of this nucleotide analogs was also found to inhibit formation of G-quartets by telomeric sequence. Other methods for converting cancer processive telomerase to the more benign non-processive telomerase include partially cleaving the telomerase RNA. The nucleoside analogs were found to be capable of a variety of activities including mediating allosteric-like inhibition of telomerase, premature termination and shortening of telomeric DNA, destabilization of telomeric structure and function and eventually cell death. Understanding the mechanisms of telomerase modulation by the 7-deaza-nucleotides has allowed the design of new telomerase inhibitors, modulators and agents for affecting telomere structure and function. These discoveries have application in the treatment of cancer.</p>			

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APPLICATION FOR UNITED STATES LETTERS PATENT
for
METHODS AND COMPOSITIONS FOR MODULATION AND
INHIBITION OF TELOMERASE

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The government has certain rights in the invention pursuant to Grant CA67760 from the National Institutes of Health. This application is a continuation-in-art of provisional patent application No. 60/000,927 filed July 6, 1996, the entire contents of which are incorporated herein without prejudice and without disclaimer.

5

BACKGROUND OF THE INVENTION

A. Field of the Invention

The present invention relates generally to the field of molecular biology. More particularly, certain embodiments concern methods and compositions useful in modulating or inhibiting human telomerase activity. In certain embodiments, the invention concerns the use of these agents in treatment of proliferative cell disorders, particularly for cancers whose proliferation is determined by processive telomerase activity.

15

B. Description of Related Art

Telomeres play an important role in chromosome organization and stability. Human telomerase is a terminal transferase that adds TTAGGG units onto the telomere end. In general, telomerase activity is not detected in normal somatic cells leading to the implication of telomerase in cancer and the impetus to develop agents that selectively target telomerase activity.

20

1. Genomic Instability of Cancer Cells

One of the general characteristics of cancer cells is genomic instability. Though it is still unclear what causes this instability, a hypothesis gaining increasing attention is that free chromosome ends, either from chromosome breakage or from loss of the telomere sequences which cap the ends, are prone to illegitimate recombination events. Thus, telomeres provide stability to the chromosomes. However, there appears to be a gradual loss of telomere sequences with each cell division, perhaps because of the end-

25

replication problem. Tumor cells have shortened telomeres, but they also possess greatly elevated levels of the enzyme telomerase to overcome the end-replication problem, while normal cells do not. Thus, telomerase is an attractive target for new anti-cancer agents because of the expected selectivity for neoplastic cells.

5

2. Telomeres

Telomeres consist of simple DNA repeats at the end of eukaryotic chromosomes and the proteins that bind specifically to those sequences in whole cells (Blackburn, 1991; Zakian, 1989). Telomeric DNA sequences and structures are conserved among
10 widely divergent eukaryotes. The essential telomeric DNA consists of a stretch of a G-rich tandemly repeated sequence. Human and other vertebrate telomeres are based on TTAGGG repeat units. The telomere provides a protective "cap" for the end of the chromosome. Broken chromosomes and free DNA ends are susceptible to end-to-end fusion leading to dicentric, ring or other unstable chromosome forms, and to
15 exonucleolytic degradation (Haber, 1984; Mann, 1983; McClintock, 1941; McClintock, 1942; Roth, 1988). By protecting against these events, telomeres prevent loss of genetic information from sub-telomeric regions of the chromosome.

Telomeres, the ends of eukaryotic chromosomes, are composed of tandemly repeated guanine-rich sequences which have an important role in chromosome
20 organization and stability. However, due to the nature of DNA synthesis, the 5' ends of telomeres shorten with each round of replication leaving a 3' overhang that is subject to degradation. This has been described as the "end-replication" problem of linear chromosomes (Watson, 1972; Olovnikov, 1973). The end-replication problem can be overcome by addition of nucleotides to the 3' end of the telomere. A telomere terminal
25 transferase (telomerase) activity was initially discovered in *Tetrahymena* (Greider & Blackburn, 1985). Telomerase activity has since been found in other ciliates (Zahler & Prescott, 1988; Shippen-Lentz & Blackburn, 1989), *Xenopus* (Mantell & Greider, 1994), yeast (Cohn & Blackburn, 1995), mouse (Prowse *et al.*, 1993), and human cells (Morin,

1989). Telomerase is a ribonucleoprotein in which the internal RNA component serves as a template for directing the appropriate telomeric sequences onto the 3' end of a telomeric primer. The cloning (Greider & Blackburn, 1989) and secondary structure determinations of the *Tetrahymena* telomerase RNA have determined the template
5 portion of the RNA which has suggested a model for the mechanism of telomerase activity. Telomerase is thought to act by: 1) Telomerase binding to the 3' single-stranded overhang of the telomere (TTAGGG in humans) which base pairs with the complementary bases of the RNA component of telomerase, 2) Nucleotide addition onto the 3' end of the telomere by telomerase using its RNA component as a template, and 3)
10 Dissociation of the newly synthesized telomeric DNA from the RNA template and repositioning to allow for the next round of polymerization. This last step is called the translocation step.

The variety of secondary structures formed by the guanine -rich telomeric sequences involving G-quartets or hairpins (Guschlbauer, 1990; Williamson, 1994) may
15 have an affect on telomerase activity. For example, there is evidence that the G-tetraplex structures formed by telomeric sequences may hinder initial telomerase binding (Zahler *et al.*, 1991). On the other hand, it has been proposed that G-tetraplex formation may actually facilitate the translocation step.

G-quartet structures may also have a role in telomere function. For example, it has
20 been shown that a variety of proteins will preferentially bind to G-quartet structures (Williamson, 1994). Also, the interaction between guanine-rich DNA strands may be involved in the association of chromosomes seen in cells in the presence of varying concentrations of Na^+ (Diaz & Lewis, 1975). The function of chromosomal association is unknown but it has been proposed that it is important in such functions as homologous
25 pairing involved in meiosis (Sen & Gilbert, 1988). Recently, a yeast nuclease (*Kem1p*) was found to specifically recognize and cut only G-quartet structures (Liu & Gilbert, 1994). Deletion of this enzyme was shown to cause telomere shortening, cellular

senescence, and blockage in the pachytene stage of meiosis in yeast (Bahler *et al.*, 1994; Tishkoff *et al.*, 1995; Liu *et al.*, 1995).

Another possible function of telomeres has sparked a great deal of interest in cancer research. It has been recently proposed that telomere length may serve as a "mitotic clock" (Harley, 1995; Shay, 1995). Normal cells in which telomeres shorten to a critical length become senescent (Allsopp *et al.*, 1992; Harley, 1991). In contrast, immortal cancer cells have an unlimited replicative capacity. Due to the findings that telomerase activity is present in a variety of tumor cells, (Chadeneau *et al.*, 1995; Counter *et al.*, 1994; Counter *et al.*, 1995; Kim *et al.*, 1994) it appears that activation of telomerase is one link to cellular immortality. This makes inhibition of telomerase an ideal strategy for anti-cancer therapy. A number of nucleoside reverse transcriptase inhibitors show anti-telomerase activity in human and *Tetrahymena* (Strahl & Blackburn, 1994; Strahl & Blackburn, 1996).

3. Chromosome End Replication Problem

Telomeres play a critical role in allowing the end of the linear chromosomal DNA to be replicated completely without the loss of terminal bases at the 5'-end of each strand. Watson (1972) and Olovnikov (1971, 1973) independently described the "end-replication" problem, *i.e.*, the inability of DNA polymerase to replicate fully the ends of a linear DNA molecule. All known DNA polymerases require a primer to initiate polymerization that proceeds in 5' → 3' direction. After degradation of the RNA primers, filling-in of internal gaps, and ligation events, the parental strand remains incompletely copied. Thus, in the absence of mechanisms to overcome the end-replication problem, the 5' end of the newly synthesized DNA in each duplex is shortened following every round of DNA replication. The 3' single stranded overhang, if not degraded, is converted to a double stranded deletion in the subsequent generation.

4. Telomerase

Immortal cells appear to overcome the end-replication problem by using telomerase to add telomeric DNA repeats to chromosomal ends. Because its mechanism of action involves the copying of an RNA template into DNA, telomerase can be classified as a reverse transcriptase. However, unlike typical reverse transcriptases from retroviruses or lower eukaryotes, it is a ribonucleoprotein that contains its own RNA template as an integral part of the enzyme (Blackburn, 1992). The RNA moiety of telomerase from various ciliates has been cloned and sequenced. For example, the *Tetrahymena* telomerase RNA moiety is a 159-nucleotide RNA in which a 3'-CAACCCCAA-5' (SEQ ID NO:1) sequence serves as the template for the synthesis of TTGGGG repeats (Greider, 1989). In *Euplotes* telomerase, a 15 nucleotide portion, 3'-CAAAACCCCAAACC-5' (SEQ ID NO:2) of a 191 nucleotide RNA was found that could serve as a template for the synthesis of TTTTGGGG repeats (Shippen-Lentz, 1990). It is of interest to note that the *Euplotes* 191 nucleotide RNA and the *Tetrahymena* 159 nucleotide RNA share little overall primary sequence similarity. However, despite their divergent primary structures, the *Euplotes* 191-nucleotide RNA, the telomerase RNAs from *T. thermophila*, and these of other ciliates can all be folded into similar secondary structures with the putative telomeric template domains for each RNA lying in a corresponding position (Shippen-Lentz, 1989). Studies indicate that human telomerase also contains an endogenous RNA as template (Morin, 1989). The RNA component of human telomerase has been cloned (Feng, 1995).

Telomerase activity from human cells possesses a number of characteristics. First, in the presence of a G-rich human telomeric primer (TTAGGG)₃ (SEQ ID NO:3), TTP, dATP and [α -³²P]dGTP, a ladder consisting of bands spaced six bases apart will form (Morin, 1989). Second, since telomerase contains an RNA component, telomerase activity can be obliterated in the presence of RNase A. It has been shown that the bands formed in the presence of excess cold nucleotides TTP and dATP and limiting amounts

of [α - 32 P]dGTP (1.56 μ M) are indicative of a pause site at the first guanine in the repeating unit of TTAGGG (Morin, 1989).

Evidence to date indicates telomerase is present in tumor cells but not in normal somatic cells. Thus, telomerase is an attractive novel drug target because there is a strong possibility for selectivity. Strahl and Blackburn (1994) has reported that several chain-terminating inhibitors (arabinofuranosyl-guanine triphosphate, Ara-GTP and 2', 3'-dideoxyribofuranosyl guanine triphosphate, ddGTP) efficiently inhibit *Tetrahymena* and human telomerase (Strahl and Blackburn, 1996).

5. Telomere/Telomerase in Cellular Senescence, Immortalization and Cancer

Early studies on human chromosome ends demonstrated that somatic (peripheral blood) telomeres appeared significantly shorter than germline (sperm) telomeres from the same individual (Cooke, 1986; Allshire, 1988; de Lange, 1990). It is now generally known that in most (if not all) somatic tissues, chromosomes gradually lose their terminal telomere sequence with each cell division (Harley, 1990; Hastie, 1990; Lindsey, 1991; Allsopp, 1992; Shay, 1993; Vaziri, 1993; Klingelhutz, 1994). In contrast, sperm telomeres increase in length with donor age, indicating that telomeres are actively maintained and even elongated in the germline (Allsopp, 1992). One explanation for this difference is that telomerase is active in germline cells but somehow is turned off in normal somatic tissues. This hypothesis has some support as no detectable telomerase activity has been found in extracts of embryonic kidney cells, or normal ovarian epithelium (Counter, 1992; Counter, 1994).

Whether telomere shortening has an impact on the proliferative activity of somatic cells remains unknown. The minimum telomere length required for maintaining full telomere function has not been fully established. Some evidence suggesting that telomere shortening could play a role in cellular aging comes from the analysis of primary human fibroblasts grown in culture (Harley, 1990; Allsopp, 1992). These cells lose

approximately 50 bp per doubling and eventually stop dividing at a senescence stage call M1. Cells arrested at the M1 stage can be rescued by a variety of viral agents (Counter, 1992; Ide, 1984; Wright, 1989; Radna, 1989). The virally transformed cells continue to divide for as many as 50 cell divisions before they face another crisis, called M2, which is characterized by a balance of cell divisions and cell death (Counter, 1992). Cells that have bypassed the M1 arrest continue to lose their telomeric DNA, resulting in much shorter telomeres. An average telomere length of approximately 1.5 kbp or less as the cell approaches M2 may not contain sufficient telomere sequence to sustain normal telomere function.

10 Occasionally, virally transformed cultures yield immortal cells that have overcome the M2 crisis. Interestingly, unlike the senescent cells, the telomere of the immortal cell lines is stabilized by re-activation of the enzyme telomerase. Counter has postulated that telomerase activation is an obligatory step in the immortalization of human cells (Counter, 1992). In support of this hypothesis, telomerase activity was
15 recently detected in metastatic human ovarian carcinoma cells but not in normal control cells, including healthy ovarian epithelium (Counter, 1994).

6 Telomerase Biochemistry

20 Alternatively, nucleotide analogs that are incorporated into telomeres by the action of telomerase may interfere with the function of the telomeres. For example, some nucleotide analogs so incorporated may block the ability of telomeres to form G-quarted or G-hairpin structures, thereby rendering the telomeres unable to be recognized by protein which specifically bind these structures. In the current model for the mechanism
25 of telomeric DNA synthesis by telomerase (Blackburn, 1990), the 3' nucleotides of the overhang region of the chromosome terminus base-pair with a telomere-complementary sequence in the telomerase RNA. Next, the chromosomal end is extended using the RNA as a template, resulting in the addition of six telomeric nucleotides. Then, the extended

DNA terminus unpairs from its RNA template and is repositioned on the 3' portion of the template, becoming available for another round of elongation by telomerase.

One of the most striking features of the telomerase reaction is that it involves not only copying of an internal template, but also an efficient translocation event which occurs after the last [5' most] residue of the template has been copied into DNA. The translocation step has been deduced from the processive nature of the telomerase reaction in a cell-free assay. Thus, telomerase initiates synthesis on a telomeric sequence DNA primer, and in the presence of an excess of the same primer or of a high concentration of a challenging primer, continues to elongate the first primer up to hundreds of nucleotides before dissociation (Blackburn, 1992).

Not all telomerase preparations are processive in the cell-free assay. Nonprocessive telomerase activity has been described in mouse FM3A cells (Prowse, 1993), *Tetrahymena* (Collins, 1993) and *Xenopus* (Mantell, 1994). Both processive and nonprocessive telomerases have been identified by the inventors from different cell lines. Intriguingly, the telomerase in S100 extracts of the human HeLa-S3 subline is non-processive, while the telomerase in the parental HeLa cells is processive. Whether these are two different enzymes or the same enzyme with different isoforms is currently being investigated. Whether processivity or non-processivity of the activity identified in the biochemical assay is relevant to telomerase function in whole cells remains to be elucidated. Evidence suggesting that telomerase functions nonprocessively in whole cells has been documented (Blackburn, 1992).

7 Deficiencies in the Prior Art

Strahl and Blackburn (1994) have tested AZT-TP, ara-GTP and ddGTP against a non-mammalian *Tetrahymena* telomerase and human telomerase (Strahl and Blackburn, 1996). Detailed methods and agents for inhibiting telomerase have not been described. In addition, there has been no distinction made between the telomerase produced in some normal cells and the telomerase produced by cancer cell. The identification of

therapeutic compounds which have modulation or inhibitory activity against human telomerase is a desirable goal, particularly to identify compounds and develop methods of treatment of cancers in which processive telomerase contributes to the immortality and undesirable proliferation.

5 Toward this end and because of the important if not entirely understood role of telomerase in cell growth and senescence, there has been an effort to identify compounds that affect telomerase activity. Use of such compounds in controlling cell proliferation has obvious implications in treatment of malignant cancers. A goal of current medical investigation is to understand and treat cellular disorders, preferably to selectively target
10 cancer cells either by altering the telomere, the telomerase, or the enzyme structure and/or by inhibiting telomerase.

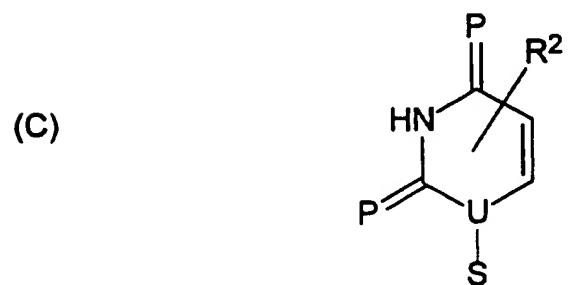
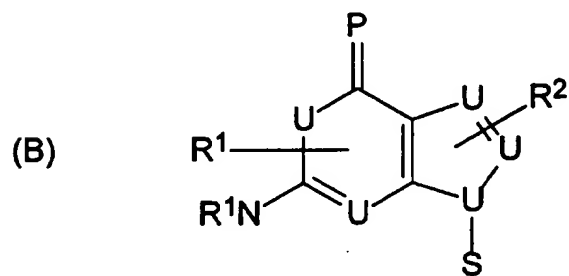
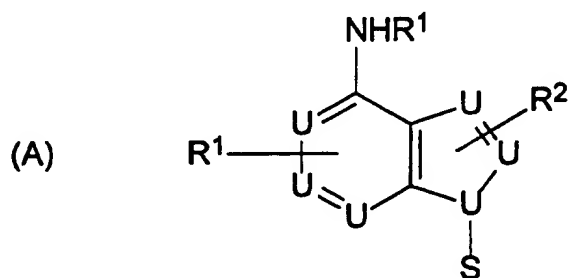
SUMMARY OF THE INVENTION

The present invention overcomes one or more of these and other drawbacks
15 inherent in the prior art by providing compositions and methods for their use in the inhibition and modulation of eukaryotic telomerase activity. More particularly, certain compositions have been shown to modify telomerase activity in cancer cells to more nearly approximate that found in normal or benign cells. Using a cell free biochemical assay, several classes of compounds, including nucleoside triphosphates and their
20 derivatives have been identified as having an inhibitory effect on human telomerase. These compounds have a surprising effect on telomerase in apparently modulating processive telomerase to a non-processive activity. These results now offer new avenues of therapy for treatment of cancers characterized by cells with processive telomerase activity.

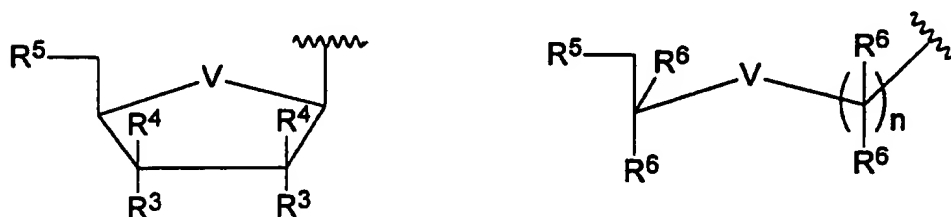
25 In the quest for finding nucleotide analogues that inhibit telomerase, 7-deaza-2'-deoxyguanosine triphosphate and 7-deaza-2'-deoxyadenosine triphosphate were found to be particularly potent inhibitors of telomerase activity. These compounds were originally investigated based on the rationale that the N-7 nitrogen of purine bases is required for

- Hoogsteen base-pairing involved in secondary structures formed by telomeric sequences.
- Unexpectedly, the 7-deaza nucleotides turned out to be poor substrates for human telomerase and were effective modulators of processive telomerase. The nucleotides thus not only present a novel mode of telomerase inhibition but also are useful for the study of the role of DNA secondary structure in telomerase mechanism.

The compounds of the present invention have the general structure (A), (B) or (C)



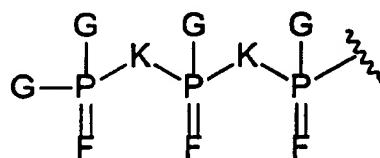
where U is independently carbon or nitrogen; R^1 is independently H, lower alkyl, or phenyl alkyl; R^2 is independently H, lower alkyl, NH_2 , halogen, azido or alkene; P is independently oxygen or sulfur; and S is an acyclic or cyclic glycosyl group represented by the formulae:



5

where R^3 is independently H, halogen, amino, azido, or hydroxyl; R^4 is independently H or OH; V is oxygen or methylene; and R^5 is OH, $(CH_2)_n$, $PO(OR^7)_2$, $OP(O)(OR^7)_2$; R^6 is H, lower alkyl, hydroxy-substituted lower alkyl, or haloalkyl; R^7 is H, lower alkyl, CH_2OCO (branched or straight chain alkyl (C1-C8) or aryl, and R^8 is H or CO(lower alkyl) and n is 1-2;

10



where K is oxygen or methylene; G is independently oxygen, sulfur or methyl; F is independently oxygen or sulfur; and the glycosyl bond between S and U is α or β .

15

The 7-deaza compositions of the present invention found to be useful telomerase-inhibiting analogs may be modified in any of several ways. It is convenient to consider the design of analogs and derivatives in three general categories: (1) modifications of the heterobase of the nucleoside triphosphate. (2) modifications of the ribose sugar; and (3) modifications of the phosphate backbone

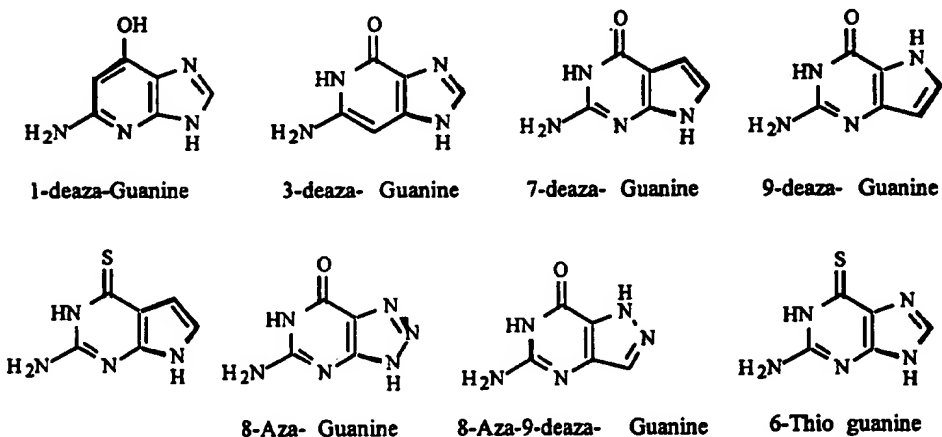
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The discovery that 7-deaza-2'-deoxynucleosides are effective in altering telomerase activity, has led to the conclusion that there are a wide range of modifications that can be made in this general class of deaza purines and pyrimidine nucleosides thus

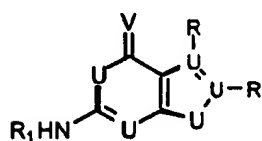
providing a plethora of compounds for selection by the practitioner in seeking to modulate telomerase activity.

Particular structures of the base portion of the nucleotide compounds contemplated by the inventors as useful for telomerase modulation or inhibition include the guanine analogs shown:

Deaza-Guanines or Aza-Guanines, 6-Thioguanine



These compounds are summarized in the generalized formula below:



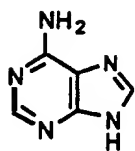
U = separately and independently C or N;

V = O or S;

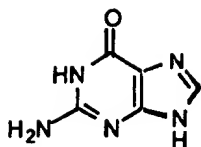
R = H, Alkyl, Alkene, Alkyne, Amino, Halogen, Azido;

R₁ = H, Alkyl, phenylalkyl

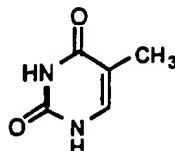
The base components of dATP, dGTP, and dTTP are Adenine, Guanine, and Thymine, respectively. The structure of adenine, guanine, and thymine is shown below:



Adenine

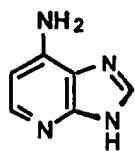


Guanine

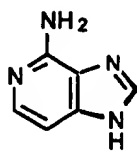


Thymine

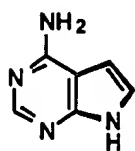
Likewise, one may use adenines, such as deaza or azoadenines:



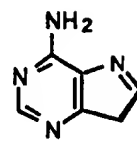
1-deaza-Adenine



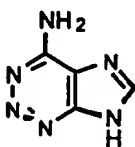
3-deaza-Adenine



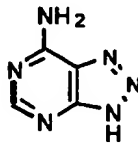
7-deaza-Adenine



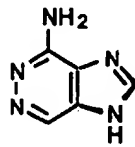
9-deaza-Adenine



5 2-aza-Adenine



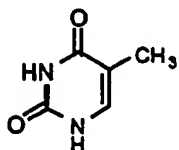
8-aza-Adenine



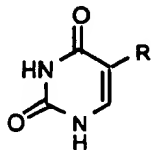
2-aza-3-deaza-Adenine

In like manner modified thymine bases may be used, including:

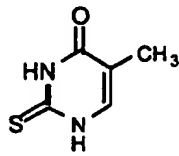
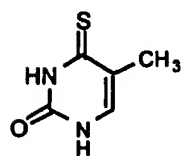
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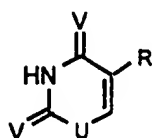
Thymine



$R = C_2H_5, C_3H_7, F, Cl, Br, I$



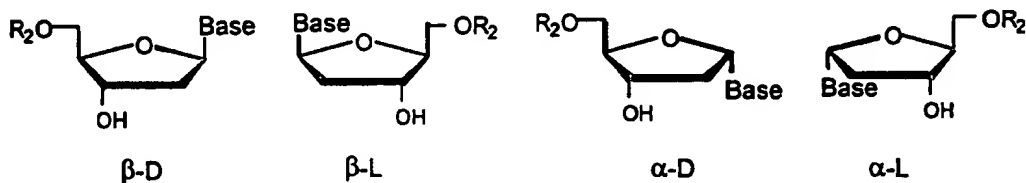
The following general formula summarizes these modifications on the thymine base.



U = separately and independently C or N;
V = O or S;
R = H, Alkyl, Alkene, Alkyne, Amino, Halogen, Azido;

5

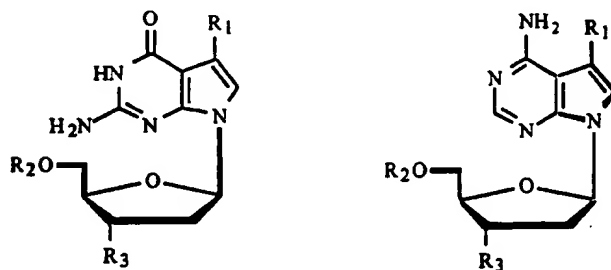
The bond between the base and the sugar moiety may be alpha or beta. The natural nucleoside is the β -D form. Nonnaturally occurring forms include β -L, α -D and α -L.



10

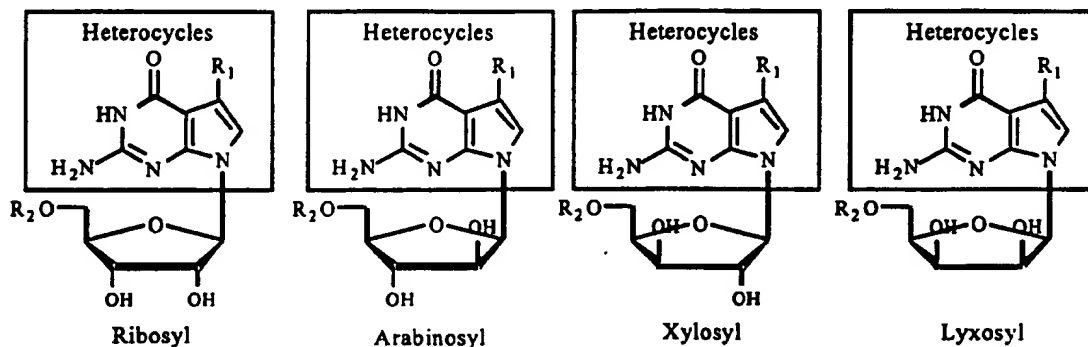
Further examples of nucleotide analogs with sugar modification include the 2'-Deoxy-3'-deoxy-3'-substituted nucleosides (or nucleotides):

15



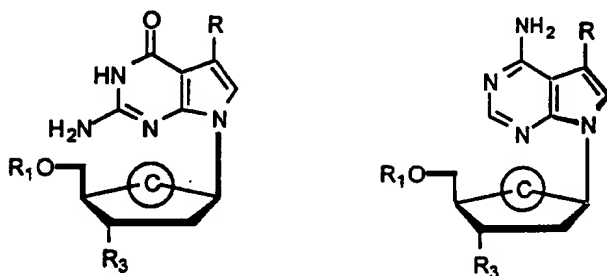
R_1 = CH_3 , C_2H_5 , etc, and Halogen, F, Cl, Br, I etc
 R_2 = H (nucleoside), or any of the modified nucleoside triphosphates
 R_3 = H, F, Cl, NH_2 , N_3

Of course the sugar moiety need not be limited to any particular sugar and several sugars are contemplated as suitable including in addition to 2'-deoxyribose, ribose, arabinose, xylose, and lyxose

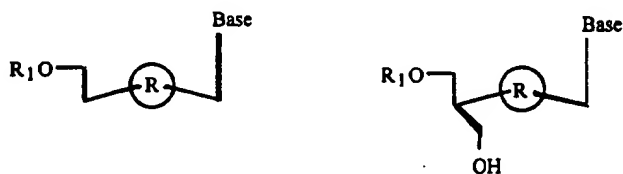


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Carbocyclic compounds may be substituted for the natural sugars:



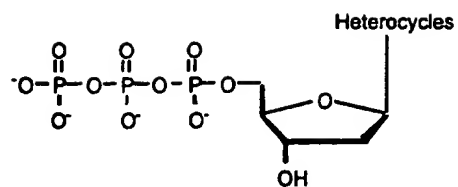
10 Acyclic compounds would also be expected to substitute for the sugar residue.



$R = \text{O, S, CH}_2, \text{ or OCH}_2$

$R_1 = \text{H (nucleoside), or any of the modified nucleoside triphosphates}$

The following modified phosphate groups may be attached to appropriate purine or pyrimidine nucleosides, particularly to 7-deaza-2'-deoxyadenosine and 7-deaza-2'-deoxyguanosine residues.



Heterocycles

- Guanine

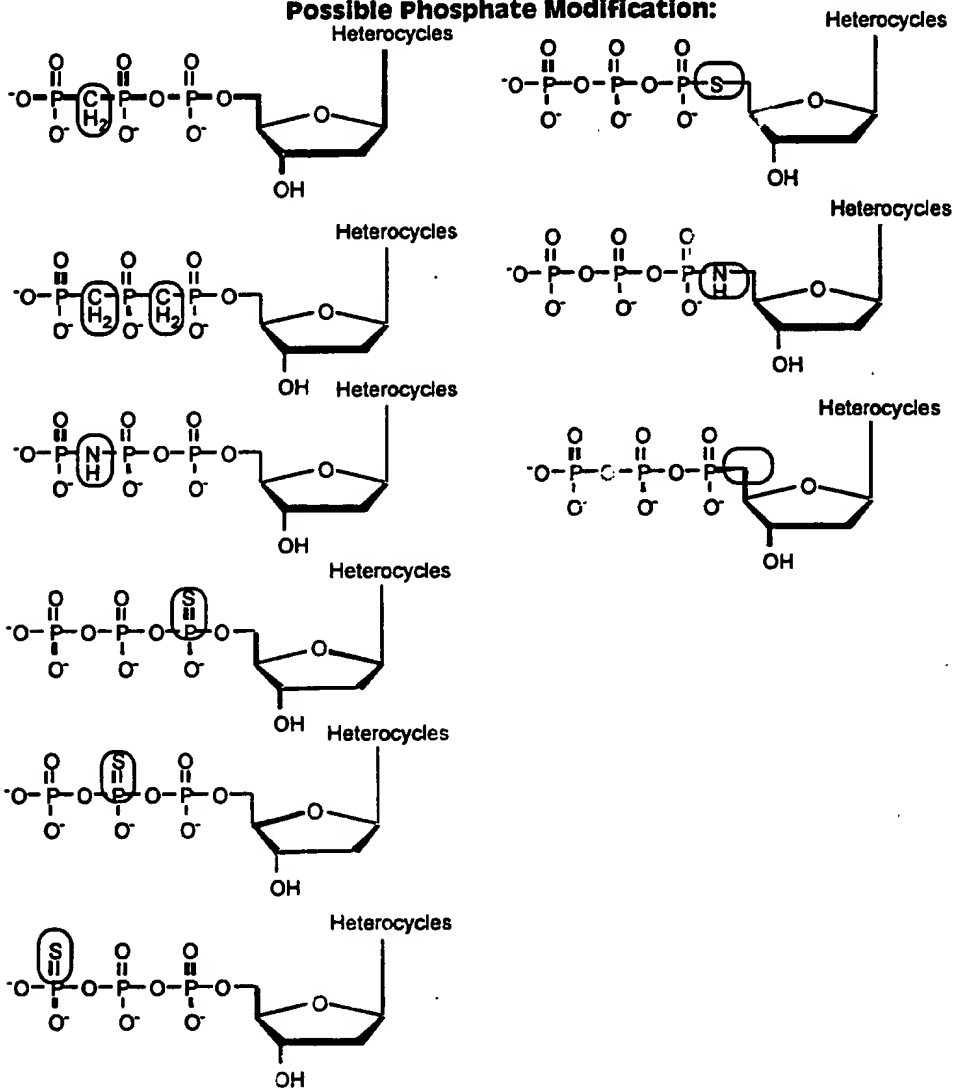
- 7-deazaguanine

- **adenine**

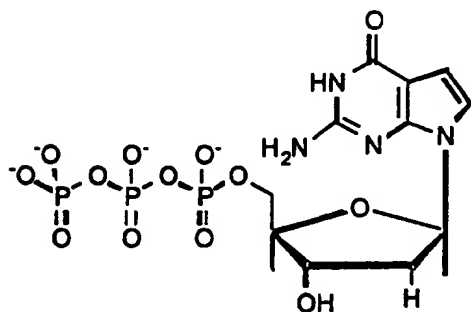
- 7-deazaadenine

etc

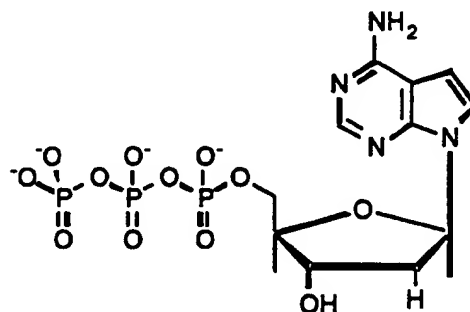
Possible Phosphate Modification:



Particularly preferred 7-deaza compounds useful for the practice of the present invention are 7-deaza-dGTP and 7-deaza-dATP, having the structures shown:



NA022; 7-Deaza-dGTP

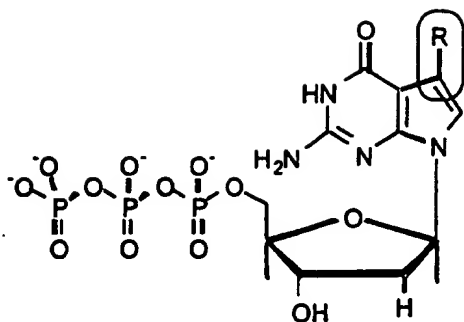
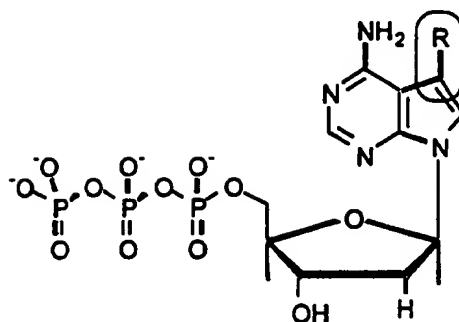


NA023; 7-Deaza-dATP

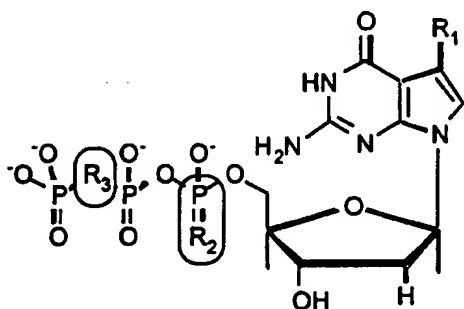
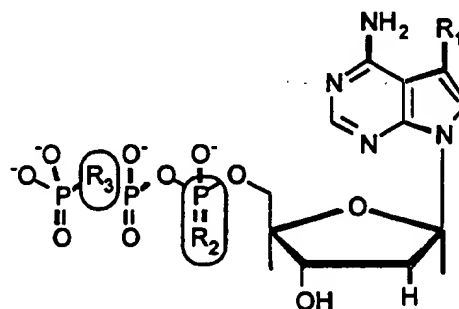
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These analogs have been shown to affect telomerase activity as follows: (i) Both compounds inhibit telomerase in a dose-dependent manner; (ii) 7-deaza-dGTP and 7-deaza-dATP are incorporated into telomeric DNA by telomerase. 7-deaza-dATP can promote non-progressive activity by telomerase. However, incorporation of 7-deaza-dATP or 7-deaza-dGTP results in a telomeric ladder that is prematurely shortened; and (iii) Substrate inhibition (or allosteric inhibition) of 7-deaza-dGTP or 7-deaza-dATP is observed at a high concentration.

10
15

**7-Substituted-7-Deaza-dGTP****7-Substituted-7-Deaza-dATP**

$R = \text{CH}_3, \text{C}_2\text{H}_5, \text{etc or halogen, F, Cl, Br, I}$

**7-Substituted-7-Deaza-dGTP****7-Substituted-7-Deaza-dATP**

$R_1 = \text{CH}_3, \text{C}_2\text{H}_5, \text{etc or halogen, F, Cl, Br, I}$

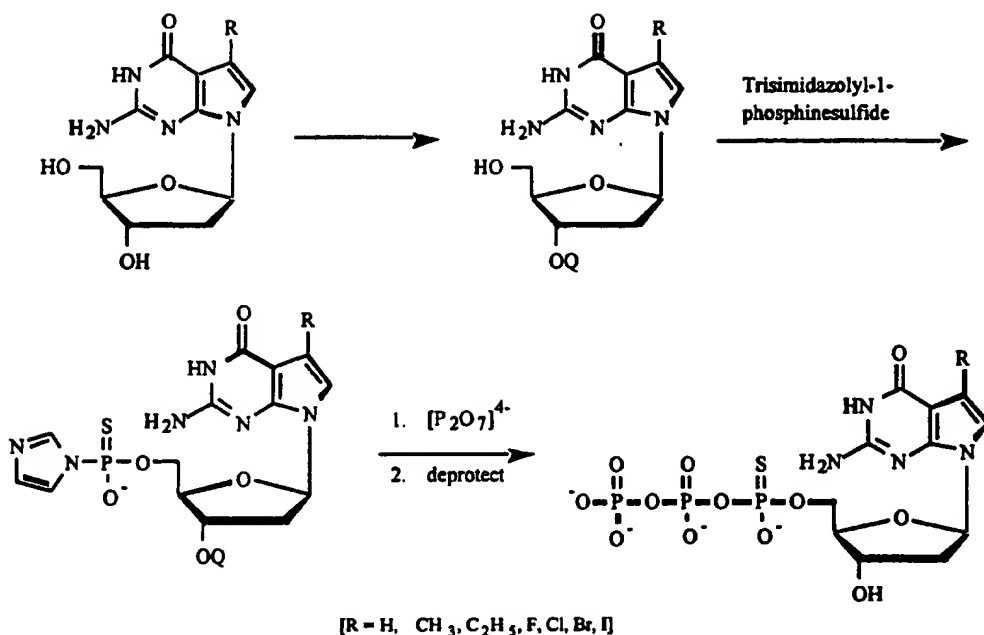
$R_2 = \text{S}, R_3 = \text{O};$
 $R_2 = \text{O}, R_3 = -\text{CH}_2-$
 or $R_2 = \text{S}, R_3 = -\text{CH}_2-$

5

Synthetic procedures for the preparation of nucleotide analogs are available and some of these are preparable by analogy to procedures found in the literature (Scheit, 1980).

A potential general synthetic pathway is outlined below for 7-substituted-7-deaza-dGTP α -phosphorothioates. Selected 7-substituted-7-deaza-dATP α -phosphorothioates could

10 be prepared in a similar manner.



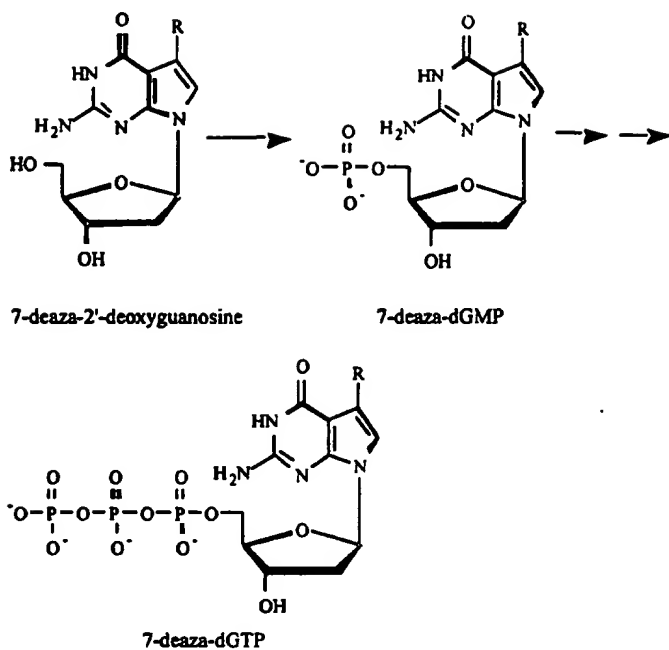
Where Q is a protecting group such as a silyl protecting group, e.g. tet-butyl-dimethyl silyl, tetrahydropyranyl, benzyl, etc.. In further aspects of the invention it has been found that human telomerase contains a 3'-5' exonuclease activity, analogous to *Tetrahymena* telomerase. Thus the synthesis of human telomeric DNA by telomerase is a balance of the 3'-5' exonuclease activity and the 5'-3' polymerase activity. In principle, synthesis of telomeric DNA can be reduced by blocking the polymerase or by stimulating the exonuclease, or both. One should be able to stimulate the exonuclease by drug interaction, thereby providing an effective method to shorten telomeres rapidly in cancer cells that produce high levels of telomerase, leading to a rapid cell death. Methods for stimulating the exonuclease include altering the interaction of dTTP and possibly other nucleotides with the telomerase. Compounds that block or limit dTTP are contemplated as useful with such a method.

Telomerase has a proof-reading-like exonuclease activity, so that incorporated modified nucleotides can be removed with no effect. The inventors contemplate that

excision of the modified nucleotides can be achieved by blocking the telomerase exonuclease with an alpha-thionucleotide. Such incorporated nucleotides, cannot easily be removed by exonuclease activity because the thio-phosphate linkage is not cleaved by exonucleases. Suitable compounds include alpha, beta and gamma thio 7-deaza
5 guanosine, adenosine and thymidine triphosphates such as for example alpha-thio-7-deaza-dGTP.

Some of these nucleotide analogs appear to also inhibit telomerase polymerization by a competition or allosteric mechanism mediated through telomerase inhibition.

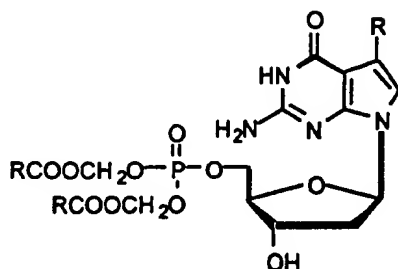
The inventors believe that 7-deaza-2'-deoxynucleoside purines and pyrimidines
10 herein described as telomerase modulators or inhibitors will be of particular use in the treatment of human cancers associated with high levels of processive telomerase. However, in order for these 2'-deoxynucleosides to exert their activity, they must be converted to triphosphates intracellularly, *i.e.*, 7-deaza-2'-deoxyguanosine must be transported into the cells and be phosphorylated by a nucleoside kinase to 7-deaza-dGMP
15 and, subsequently, further phosphorylated to 7-deaza-dGDP and 7-deaza-dGTP, respectively.



In general, phosphorylation of a nucleoside to its monophosphate is a rate-limiting step in whole cells. If the cells were unable to phosphorylate the nucleosides to monophosphates, no intracellular di- or triphosphates can be formed or identified. On the other hand, one also cannot simply incubate the cells with the phosphates (mono-, di-, or triphosphates) and expect them to transport into the cells and provide the cells with the triphosphates of nucleoside analogs. The phosphates are highly negatively charged, and therefore, will not transport into cells. Instead, the mono-, di-, or triphosphates will be dephosphorylated extracellularly by alkaline phosphorylase or 5'-nucleotidase back to the nucleoside.

In order to circumvent these problems, one can prepare prodrugs of monophosphates in which the negatively charged phosphate group is functionalized. The prodrug of monophosphates will then contain no charged groups and can be efficiently transported into cells. Once it has entered the cells, the protective group is hydrolyzed by esterase, and nucleoside monophosphate is released. Subsequently, phosphorylation of

the liberated monophosphate will produce the desired nucleoside triphosphate. The general structure of the prodrugs is shown below:



The use of this type of prodrug approach to deliver nucleotides to cells has been described (Farquhar *et al.*, 1983; Sastry *et al.*, 1992). Such compounds can enter cells and release the monophosphates which can be further phosphorylated to the corresponding triphosphates.

The present invention has identified one of the most potent telomerase inhibitors, 7-deaza-2'-deoxyguanosine-5'-triphosphate. The IC_{50} value (6.8 μM) is at least 50-times more potent than AZT-TP. 7-deaza-2'-deoxyguanosine-5'-triphosphate lacks the 7-nitrogen atom which is essential for the formation of G-quartets or hairpins. If 7-deaza-2'-deoxyguanosine-5'-triphosphate is incorporated into telomeres, further telomere elongation may be prevented.

Several nucleoside triphosphate analogs with phosphate backbone modifications were demonstrated to be potent telomerase inhibitors. These phosphate backbone modifications include (1) thiophosphate, where the (α) P=O bond is replaced with a P=S bond, *e.g.*, NA013 (IC_{50} = 81.5 μM) and (2) phosphonate, where the oxygen atom joining the β and γ phosphate (P) atom is replaced with a methylene ($-CH_2-$) group *e.g.* NA014 (IC_{50} = 102 μM). (One of the most potent inhibitors identified was 7-deaza-2'-deoxyguanosine-5'-triphosphate (NA022) with IC_{50} value of 6.8 μM . 7-deaza-2'-deoxyadenosine-5'-triphosphate (NA023) also inhibits telomerase activity with an IC_{50} value of 78.5 μM);

It is now possible to define the structure-activity relationship of nucleoside/nucleotide analogs as inhibitors and modulators of telomerase, thus allowing additional specific inhibitors and modulators of the enzyme to be identified. The design and synthesis of potent telomerase inhibitors based on these studies provide an array of telomerase modulating drugs to be used in the treatment of proliferative cell disorders, and particularly those involving cancers characterized by high levels of processive telomerase.

Useful telomerase-inhibitory compounds are not believed to be limited in any way to the specific compounds or nucleotide analogs and derivatives specifically disclosed herein. In fact, it may prove to be the case that the most useful pharmacological compounds designed and synthesized in light of this disclosure will be second generation derivatives or further-chemically-modified compositions.

Where telomerase-containing cells are located within an animal, a pharmaceutically acceptable composition of the telomerase inhibitor may be administered to the animal in an amount effective to modify the telomerase activity of the target cell. In terms of inhibiting telomerase activity in tumor cells, this is contemplated to be an effective mechanism by which to treat cancer that will have very limited side effects.

An embodiment in which the compositions of the present invention find particular utility is the treatment of cell proliferative disorders, and in particular human tumors characterized as having processive telomerase. The utilization of telomerase inhibitors (which either directly inhibit the telomerase activity or indirectly incorporate into telomere and thus prevent telomere further elongation) will lead to progressive telomere shortening in tumors where telomerase is active. Once the telomere length shortens to a critical length (ca 2 kb), the tumor will go into crisis and eventually die. These telomerase inhibitors will have little or no effect on the normal somatic cells because telomerase activity in normal cells is generally low or undetectable.

It is believed that the disclosed compounds will be useful for potentially treating a patient after surgical removal of a tumor. The patient would be treated with non-cytotoxic doses of nucleoside/nucleotide analogs for a prolonged period of time to

prevent the recurrence of micro-metastasis. Alternatively, effective treatment of invading pathogens susceptible to telomerase inhibition are also contemplated, as are applications in treating age-related disorders such as atherosclerosis and osteoporosis.

There are several methods contemplated by the inventors for the delivery of such telomerase inhibitors into cells. In one embodiment, cells are provided with the corresponding nucleoside analogs, and subsequent cellular metabolism converts the nucleosides into nucleoside mono-, di- and tri-phosphates. In another embodiment, formulations of specific telomerase inhibitors are prepared in vehicles which protect the nucleotide from phosphatase degradation and facilitate the transport of nucleotides. In the case of the latter, a preferred method for delivery would be that of liposome-mediated delivery. In yet another embodiment, one could prepare pro

The therapeutic potential for liposome-mediated transfer of such telomerase inhibitors into human cells is well known to those of skill in the art. Based on existing evidence which shows that the systemic injection of cationic liposome complexes into animals is non-toxic (Stewart *et al.*, 1992), the inventors contemplate the use of such liposome-mediated methods for introducing the compositions disclosed herein into animal subjects.

Liposomes have been used successfully with a number of cell types that are normally resistant to transfection by other procedures including T cell suspensions, primary hepatocyte cultures and PC 12 cells (Chang and Brenner, 1988; Muller *et al.*, 1990). Liposomes have been used effectively to introduce drugs (Heath *et al.*, 1986; Storm *et al.*, 1988; Balazsovits *et al.*, 1989), radiotherapeutic agents (Pikul *et al.*, 1987), and enzymes (Imaizumi *et al.*, 1990; Imaizumi *et al.*, 1990) into a variety of cultured cell lines and animals. In addition, several successful clinical trials examining the effectiveness of liposome-mediated drug delivery have been completed (Lopez-Berestein *et al.*, 1985; Coune, 1988). Furthermore, several studies suggest that the use of liposomes is not associated with autoimmune responses, toxicity or gonadal localization after systemic delivery Nabel *et al.*, 1992; Mori and Fukatsu, 1992).

Introduction of the liposome-telomerase inhibitor complex may be by injection, either systemically into peripheral arteries or veins (including the carotid or jugular vessels), or directly into specific tissues to be targeted. Such liposome formulations are commercially available, *e.g.*, 1:1 (w:w) mixture of the cationic lipid *n*-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE) may readily be employed for such liposome formulations.

Based on the inventors' discovery of a method to modify or inhibit telomerase activity, it is contemplated that several classes of compounds will be useful. It will be desirable to determine which analogs and derivatives will be most suitable for particular treatments; for example, depending on the type of cancer cell present and particularly the amount and activity of telomerase present. A related aspect of the invention is the discovery that certain allosteric interactive agents will alter or inhibit telomerase activity. Nucleotides such as 7-deaza dGTP and 7-deaza dATP, are capable of allosterically inducing a reduction in telomerase polymerizing activity. The inventors have demonstrated that other nucleotides such as dGTP will not induce this effect, indicating the importance of the 7-deaza modification. This now provides a new method of modulating telomerase for the treatment of cancer by designing a series of 7-deaza compounds that will allosterically bind with telomerase with varying effects on modulating telomerase activity. Highly processive telomerase for example may require stronger inhibitors or modifications to the nucleotide analog that change allosteric interactions or are less efficiently incorporated into the telomere.

BRIEF DESCRIPTION OF THE DRAWINGS

25

The drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Telomerase activity from HeLa (processive) versus UA 21 (non-processive) cells. RNase prevents telomerase activity.

5 FIG. 2. Phosphorylated metabolites of AZT may be identified and quantitated by HPLC.

FIG. 3. Inhibition of telomerase ladder with 7-deaza-dGTP (A) or dGTP (B) in the presence of 1.56 μ M [α -³²P]dGTP containing 1 mM dTTP, 1 mM dATP, 1 μ M (TTAGGG)₃, 20 μ l S100, and from left to right: 0 (C), 1, 2.5, 5, 7.5, 10, 25, 50, 75, 100 μ M 7-deaza-dGTP or dGTP.

Inhibition of telomerase ladder with 7-deaza-dATP (C) or dATP (D) in the presence of 3.12 μ M [α -³²P]dATP, containing 1 mM dTTP, 1 mM dGTP, 1 μ M (TTAGGG)₃, 20 μ l S100, and from left to right: 0 (C), 1, 2.5, 5, 7.5, 10, 25, 50, 75, 100 μ M 7-deaza-dATP or dATP.

15 FIG. 4. (A) Inhibition of telomerase ladder with 7-deaza-dGTP in the presence of 3.12 μ M [α -³²P]dATP, containing 1 mM dTTP, 1 mM dGTP, 1 μ M (TTAGGG)₃, 20 μ l S100, and from left to right: 0 (C), 0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0 mM 7-deaza-dGTP

20 (B) Inhibition of telomerase ladder with 7-deaza-dATP in the presence of 1.56 μ M [α -³²P]dGTP containing 1 mM dTTP, 1 mM dATP, 1 μ M (TTAGGG)₃, 20 μ l S100, and from left to right: 0 (C), 0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0 mM 7-deaza-dATP.

25 FIG. 5A 7-deaza-dGTP as a telomerase substrate with 3.12 μ M [α -³²P]dATP, 1 mM dTTP, 1 μ M (TTAGGG)₃, 20 μ l S100 from left to right: 1 mM dGTP (C), and 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0 mM 7-deaza-dGTP. The arrows point to the triplet band most prominent at 0.75 and 1.0 mM 7-deaza-dGTP.

FIG. 5B 7-deaza-dGTP as a telomerase substrate with 3.12 μM [$\alpha\text{-}^{32}\text{P}$]dATP, 1 mM dTTP, 1 μM (TTAGGG)₃, 20 μl S100, 1 mM 7-deaza-dGTP without (-) or (+) with RNase A at 0.125 $\mu\text{g}/\mu\text{l}$. The arrows point out the triplet bands.

5

FIG. 5C dGTP as a telomerase substrate with 3.12 μM [$\alpha\text{-}^{32}\text{P}$]dATP, 1 mM dTTP, 1 μM (TTAGGG)₃, 20 μl S100 from left to right: 1 mM dGTP (C), and 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0 mM dGTP.

10 **FIG. 6A** 7-deaza-dATP as a telomerase substrate with 1.56 μM [$\alpha\text{-}^{32}\text{P}$]dGTP, 1 mM dTTP, 1 μM (TTAGGG)₃, 20 μl S100 from left to right: 1 mM dATP (C), and 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0 mM 7-deaza-dATP.

15 **FIG. 6B** 7-deaza-dATP as a telomerase substrate with 1.56 μM [$\alpha\text{-}^{32}\text{P}$]dGTP, 1 mM dTTP, 1 μM (TTAGGG)₃, 20 μl S100, 1 mM 7-deaza-dATP without (-) or with (+) RNase A at 0.125 $\mu\text{g}/\mu\text{l}$.

20 **FIG. 6C** dATP as a telomerase substrate with 1.56 μM [$\alpha\text{-}^{32}\text{P}$]dGTP, 1 mM dTTP, 1 μM (TTAGGG)₃, 20 μl S100 from left to right: 1 mM dATP (C), and 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0 mM

FIG. 7. Telomerase activity with 1 mM dTTP, 1 μM (TTAGGG)₃, 20 μl S100, 1.56 μM [$\alpha\text{-}^{32}\text{P}$]dGTP 1 mM dATP (dGTP*) or 3.12 μM [$\alpha\text{-}^{32}\text{P}$]dATP and 1 mM dGTP (dATP*). Reactions contained either no RNase A (-) or 0.125 $\mu\text{g}/\mu\text{l}$ of RNase A (+).

25

FIG. 8. Temperature dependence of the ^1H NMR spectrum of the human telomeric DNA sequence d(GGTTAGGGTTAG) in NaCl- and KCl-containing buffers.

FIG. 9. Temperature dependence of the ^1H NMR spectrum of the human telomeric DNA sequence d(GGTTAGG*GTTAG) where G* is 7-deaza-2'-deoxyguanosine.

FIG. 10 Incorporation of NA004 by Human Cancer Telomerase.

5

FIG. 11 Incorporation of NA006 by Human Cancer Telomerase.

FIG. 12 Lack of Incorporation of NA007 by Human Cancer Telomerase.

10 **FIG. 13** Incorporation of NA013 by Human Cancer Telomerase.

FIG. 14 Incorporation of NA014 by Human Cancer Telomerase.

FIG. 15 Lack of Incorporation of NA020 by Human Cancer Telomerase.

15

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides several nucleoside triphosphates which are capable of inhibiting mammalian telomerase activity. The compounds show differential inhibition against telomerase in S100 extracts of transformed human fetal kidney tumor 20 293 cells (a processive telomerase) versus that of hamster UA21 cells (non-processive telomerase). This observation has prompted the investigation of nucleoside/nucleotide analogs which are useful in affecting telomere/telomerase function in cells.

25 A. Telomeric DNA as a Target

While inhibition of telomerase represents one way of interfering with the function of cellular telomeres, providing DNA reactive drugs which damage telomeric DNA itself is a possible complementary approach.

Damage to telomeres is more detrimental to rapidly growing (*i.e.* tumor) cells than to normal cells. Given the significance of telomeres, compromised telomere integrity results in incomplete replication of telomeric DNA, disturbed chromatin structure and, eventually, cell death. An attempt to replicate telomeres with unrepaired
5 lesions may further amplify the initial damage. Thus, tumor cells that have very short telomeres would be the most susceptible to incomplete telomere replication. Moreover, lesions in telomeric DNA may be more lethal in those tumor cells that require telomerase to actively maintain their already shortened telomeres.

Because of the presence of highly repetitive sequences (TTAGGG)_n, it is possible
10 that DNA-reactive agents with a preference for A/T or G/C may damage telomeric DNA to a different extent than other regions of the chromosome. Also, the repair of damage in telomeres is likely to be slower than repair in the rest of the genome (Kruk and Bohr, 1993).

Conventional DNA-reactive antitumor drugs may affect telomeres in addition to
15 inducing damage to other regions of the genome. Lesions in telomeres may disturb telomerase action. For example, bifunctional alkylating agents may form crosslinks between telomeric DNA and telomerase protein and/or RNA components. Such adducts will probably abrogate enzyme activity. Conversely, inhibition of telomerase may enhance lethality of drug-induced DNA lesions in other regions. By examining the
20 effects of DNA damaging drugs on telomeres one may determine which lesions in telomeres can be tolerated by cells and at what maximal levels, how lesions repaired in telomeres compare to other regions of DNA, and which drugs have potential for preferential targeting of telomeres.

25 B. Non-Processive/Processive Telomerase Activity

The non-processive telomerase activity produced by normal cells is distinctly different from the processive telomerase activity produced by cancer cells. Processive telomerase aggressively synthesizes telomeric repeats. This type of activity is necessary for maintaining or growing telomeres in cancer cells. Telomere maintenance allows

cancer cells to be immortal without the danger of chromosome instability. While normal cells do not maintain or grow their telomeres nor cell do produce a regulated and non-aggressive form of telomerase characterized by its non-processive activity. This activity appears to be necessary for certain functions in normal cells other than maintaining or growing telomeres. Unlike cancer cells, normal cells are not immortal, ostensibly due to lack of or very low levels of processive telomerase. This information indicates that a method for converting processive telomerase to non-processive telomerase in a way that would not harm normal cells but would selectively affect cancer cells.

The present invention demonstrates a method of altering processive telomerase so that it acts non-processively. This modulation causes cancer cell telomerase to more closely mimic normal cell non-processive telomerase. In general, changes to convert processive telomerase to non-processive telomerase may involve either direct changes to the telomerase complex itself or indirect methods that ultimately affect telomerase activity. Direct methods for example may involve cleaving the RNA component into partial fragments, such that only processive activity is affected. Processive telomerase activity may be modulated to nonprocessive activity by a partial digest treatment with RNase A or with other RNA cleaving agents, such as ribozymes. Similar results may be obtained by chemical modification of the RNA, or binding of agents, such as oligonucleotides.

The present invention illustrates an indirect method for converting processive telomerase to nonprocessive telomerase by allowing telomerase to incorporate 7-deaza-dGTP or related analogs into telomeric DNA. 7-deaza-dGTP and its analogs, once incorporated into the telomere, alter the telomeric secondary structure so that telomerase can no longer recognize it properly for processive telomere synthesis. Other nucleotides that can be used as substrates by telomerase, and which are incorporated into the telomere will also mediate telomere dysfunction, include alpha-thio- nucleotides. These nucleotide analogs can be incorporated in the telomere by any polymerase capable of replicating the telomere. Once in place, only the telomeric sequence is affected since the unique G-rich repeat structure of telomeric DNA is found only in the telomere.

The inventors' strategy is to provide selected nucleoside analogs that act in two ways; both to alter processive telomerase to act non-processively and to affect telomere structure so that telomere-binding proteins will no longer recognize the telomere. The telomere is thus left unprotected, resulting in chromosome instability and cell death.

5 Cancer cells will be selectively targeted because these cells depend on processive telomerase to incorporate the nucleotide analog. The more telomerase that a cell produces, the more effective the strategy. The method also has advantages over merely inhibiting telomerase, because the telomere is immediately affected and it is not necessary to wait for the telomere to shorten and become dysfunctional.

10 The disclosed method of using selected nucleoside analogs to alter telomerase activity does not inhibit telomerase but merely converts one form of telomerase to another form of telomerase.

C. Telomerase Activity in Human Tumor Cells

15 Using a telomerase assay, the inventors have successfully identified the telomerase activity in the following cell lines: 293 (a transformed human embryonic kidney cell line), HeLa and HeLa-S3 (a human cervical tumor cell line), CEM (a human leukemia cell line) and UA-21 (a subline of Chinese hamster cell CHO line). Interestingly, processive telomerase activity has been identified in the S100 extracts of
20 293, CEM, HeLa cell lines, and non-processive telomerase activity in the S100 extracts of HeLa-S3, UA-21 and WI38 cell lines (FIG. 1).

DNA ladders separated by 6 base-pairs are seen in S100 extracts of HeLa—human cervical tumor cells (FIG. 1, lane 1)—indicative of a processive enzyme; whereas only one a band is detected in S100 extracts of UA-21— a subline of Chinese hamster
25 cells (FIG. 1, lane 4)— indicative of a non-processive enzyme. Both the processive and non-processive enzyme activities are sensitive to RNase treatment (FIG. 1, lanes 2 and 5). These results indicate that the formation of the processive or non-processive DNA band(s) is RNA-dependent. The effect of RNase on telomerase activity can be prevented if RNase Guard (Pharmacia) is added to inhibit the RNase activity (FIG. 1, lanes 3 and 6).

D. Inhibition of Telomerase Activity by Selected Nucleoside Triphosphates

Selected nucleoside triphosphates were tested in one or both of the "conventional" or "modified" telomerase assays described in Methods using the human processive and the CHO non-processive telomerases. Initially, three chain-terminators were selected: 2', 3'-dideoxythymidine triphosphate (ddTTP), 3'-fluoro-2',3'-dideoxythymidine triphosphate (F-ddTTP), and 3'-azido-2', 3'-dideoxythymidine triphosphate (AZ-ddTTP) as prototype agents to investigate whether they could inhibit telomerase activity. The three compounds are dTTP analogs without a 3'-OH group for subsequent chain elongation. They may either incorporate into the telomere sequence and terminate further chain elongation or compete directly with dTTP for the substrate binding site in the telomerase reaction. In either case, telomerase activity is inhibited.

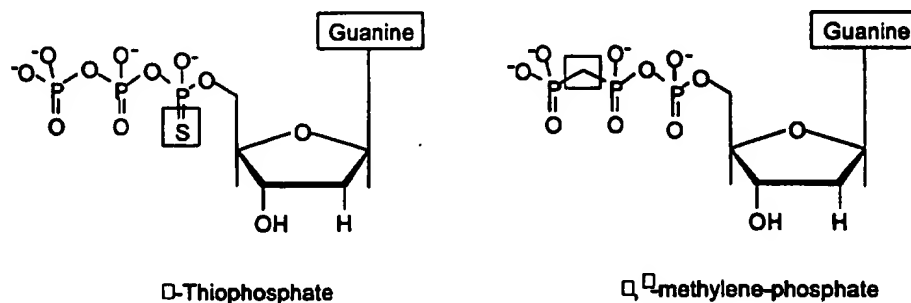
Results showed that AZ-ddTTP inhibits both the human processive and the CHO non-processive telomerase in a dose-dependent manner. The DNA ladder bands were quantitated using a Molecular Dynamic Personal Densitometer. The concentration of AZ-ddTTP required to inhibit the telomerase activity by 50% of the control is estimated to be 500 and 750 μ M against the 293 and UA-21 telomerase, respectively. In contrast, the nucleoside AZT (3'-azidothymidine), at 2 mM, has a small effect on the telomerase activity.

The results suggested that only the nucleoside triphosphate, but not nucleoside, is an inhibitor of telomerase. Inhibition studies are typically performed at concentrations of dTTP and dATP (2 mM) which could be much higher than the K_m of dTTP and dATP for telomerase. The inventors tried lower concentrations of dTTP and dATP (near the K_m ; in the μ M ranges) and showed that AZ-ddTTP is actually a more potent inhibitor of telomerase than previously estimated. It was found that ladder patterns were detectable even at dTTP and dATP concentrations as low as ~ 200 μ M. Even lower concentrations of dTTP and dATP are likely be effective with more highly purified enzyme concentrations.

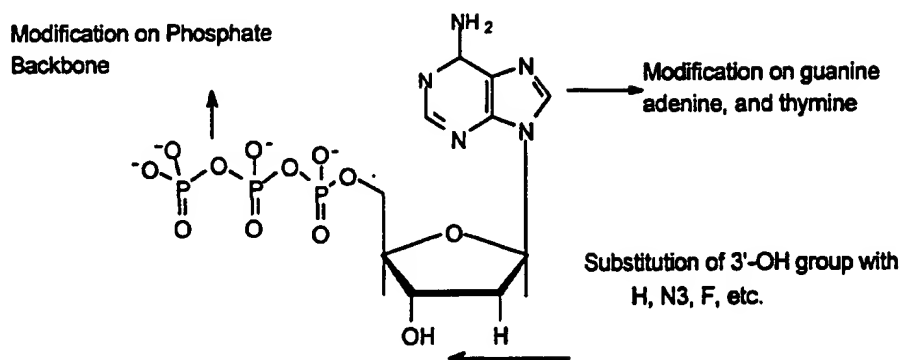
It has been shown that FddTTP, with a 3'-fluoro substituent, inhibits the non-processive CHO telomerase completely but has little effect against the processive human telomerase. The change at the 3'- position causing a distinctly different inhibitory effect on two telomerase enzymes was both surprising and quite unexpected.

5 Additional dTTP analogs were studied. A compound with a 3'-amino substitution had only a marginal effect on the human telomerase activity. However, another compound, 4-thiothymidine triphosphate, with a 4-thio group rather than a 4-keto group on dTTP, inhibited the human processive enzyme completely at 1 mM. 4-thiothymidine triphosphate has a 3'-OH group and, therefore, appears not to chain-terminate the
10 telomerase reaction when incorporated into the nascent DNA strand. This is the first observation that a dNTP analog with a heterocyclic ring modification has an effect on telomerase activity.

Selected dATP and dGTP analogs were also evaluated as potential telomerase inhibitors. 2'3'-dideoxyadenosine triphosphate (ddATP) inhibited human telomerase
15 activity at 1 mM. This was similar to results obtained with 2'3'-dideoxythymidine triphosphate (ddTTP). Two deoxyguanosine triphosphate analogs with modifications on the phosphate group were found to inhibit human telomerase activity. In one modification, the (α) P=O bond was replaced with a P=S bond to form a thiophosphate. In a second modification, the oxygen atom joining the β and γ phosphate (P) atom was replaced with a methylene ($-\text{CH}_2-$) group to form a phosphonate. The new analogs have
20 the following structure.



It is expected that modifications at the sites indicated will provide additional nucleoside triphosphate telomerase inhibitors.



5

E. Effect of AZT (3'-Azidothymidine) on Telomere Length and Formation of Dicentric Chromosomes

AZ-ddTTP is a negatively charged molecule and cannot be transported into cells. In order to study the effects of AZ-ddTTP on telomerase activity in a whole cell system, CHO cells were incubated with 800 μ M (non-cytotoxic concentration) of 3'-azidothymidine (AZT nucleoside) for several generations. The effect of AZT on the telomere length and on the formation of unstable dicentric chromosomes was determined as an indirect measurement of the effect of AZ-ddTTP on intracellular telomerase activity. An initial, gradual shortening of the telomere length was found with a concurrent increase in the number of dicentric chromosomes in the AZT-treated CHO cells. This effect was only transient; after 20 generations, the fraction of cells with dicentric chromosome decreased.

AZddTTP inhibits both the 293 and UA-21 enzymes. Studies have shown that the telomere length in cultured human cells passaged in non-cytotoxic concentrations of AZT

(azidothymidine, nucleoside) is progressively shortened. In addition, the frequency of dicentric chromosomes (measured by fluorescence *in-situ* hybridization) increased in the presence of AZT. Eventually, the cells stopped growing and apparently died.

5 **F. Intracellular Accumulation of AZT Mono-, Di-, and Tri-Phosphate Levels**

Since only AZ-ddTTP (AZT triphosphate), and not AZT, inhibits telomerase activity, studies were initiated to determine intracellular AZT triphosphate concentrations. The metabolism of AZT is species- and cell type-specific (Balzarini, 1988; Balzarini 1989). In CHO cells, using ³H-labeled AZT, it was found that the uptake
10 (transport and subsequent metabolism to its phosphate metabolites) of AZT was concentration- and time-dependent. AZT accumulates intracellularly at concentrations 2-3 times higher than the extracellular concentrations (0.25-750 μM) tested. Total AZT uptake reached a plateau in approximately 30 minutes. Subsequent HPLC analysis using a strong anion exchange column showed that the intracellular AZT, AZ-ddTMP, AZ-
15 ddTDP, and AZ-ddTTP ratios are approximately 50-60:20:15:10 following a 90 minute incubation with 5 - 25 μM of AZT (FIG. 2). It is estimated that about 200 μM of AZddTTP could be formed intracellularly when cells are incubated with 750 μM of AZT. This intracellular concentration is lower than the IC₅₀ value obtained in the cell-free assay for the inhibition of the telomerase activity.

20

G. Inhibition of Telomerase by 7-deaza-dGTP and 7-deaza-dATP in the presence of limiting amounts of dGTP and dATP.

FIGS. 3A and 3C illustrate the effect of 7-deaza-dGTP and 7-deaza-dATP on the
25 level of telomerase activity. Inhibition of telomerase activity was indicated by a reduction in the intensity of the bands with increasing concentrations of inhibitor. The degree of inhibition was determined by measuring the intensity of the bands in the telomerase ladder with densitometry. The intensities in each lane were normalized to an internal standard, ³²P 5' end-labeled (TTAGGG)₃, to compensate for differences in

processing of the products and gel loading. Intensities in all lanes were expressed as a percent of the control to eliminate variability due to differences in film exposure and enzyme preparations. The concentration of inhibitor that results in a 50% reduction in telomerase activity (IC_{50}) was determined according to the method outlined in Materials and Methods (IC_{50} values are in Table 1). The similarity of their IC_{50} values reveals that 7-deaza-dGTP is just as efficient as cold dGTP in inhibiting the formation of [α - ^{32}P]dGTP labeled telomerase ladders (FIG. 3A & FIG. 3B, Table 1). Both 7-deaza-dATP and cold dATP have a similar capacity to inhibit the formation of [α - ^{32}P]dATP labeled products (FIG. 3C and FIG. 3D, Table 1).

10

TABLE 1. Inhibition of radiolabeled human telomerase ladder by 7-deaza-dGTP, 7-deaza-dATP, dGTP, and dATP.

Inhibitor	[α - ^{32}P]dNTP (μ M)	Non-radioactive Nucleotides (mM)	IC_{50} (μ M)
7-deaza-dGTP	dGTP* (1.56 μ M)	TTP and dATP (1 mM)	11
dGTP	dGTP* (1.56 μ M)	TTP and dATP (1 mM)	5
7-deaza-dATP	dATP* (3.12 μ M)	TTP and dGTP (1 mM)	8
dATP	dATP* (3.12 μ M)	TTP and dGTP (1 mM)	10
7-deaza-dGTP	dATP* (3.12 μ M)	TTP and dGTP (1 mM)	56
7-deaza-dATP	dGTP* (1.56 μ M)	TTP and dATP (1 mM)	59

IC_{50} values were obtained according to Materials and Methods.

15 **H. Inhibition of telomerase activity by 7-deaza-dGTP and 7-deaza-dATP in the presence of excess amounts of dGTP and dATP**

7-Deaza-dGTP and 7-deaza-dATP can also inhibit telomerase activity even in excess (1 mM) dGTP or dATP respectively (FIG. 4A and FIG. 4B). The IC_{50} value of 7-deaza-dGTP as a telomerase inhibitor in the presence of 1 mM dGTP, dTTP and 3.12 μ M [α - ^{32}P]dATP was 56 μ M. The IC_{50} of 7-deaza-dATP was 59 μ M when in the presence of 1 mM dATP, TTP and 1.56 μ M [α - ^{32}P]dGTP.

20

I. 7-Deaza-dGTP and 7-deaza-dATP are telomerase substrates

Replacing dGTP with 7-deaza-dGTP in a reaction containing 1 mM dTTP and 3.12 μ M [α - 32 P]dATP resulted in formation of radiolabeled products that are sensitive to RNase A (FIG. 5A and FIG. 5B). There are important characteristics of this reaction which distinguish it from a reaction with the native dGTP. First, the total amount of products produced by 7-deaza-dGTP was much less than that produced with dGTP. Second, telomerase appears to be less processive with 7-deaza-dGTP. As the concentration of 7-deaza-dGTP increased, the products became increasingly shorter and the total amount of products decreased until there was no activity at > 1.5 mM 7-deaza-dGTP. There is no significant change in activity when dGTP is used as a substrate in the reaction with concentrations varying from 0.25 mM-2.0 mM (FIG. 5C).

Telomerase paused at significantly more sites in the presence of 7-deaza-dGTP. At a concentration of 0.5 mM 7-deaza-dGTP, there was such a large number of bands pertaining to various pause sites that a predominant pause site could not be determined. When the 7-deaza-dGTP concentration is 0.75-1 mM, a repeating triplet of pause sites was distinguishable (FIG. 4A lanes 5 and 6, FIG. 5B). These bands appeared to be two, three, and four bases smaller than that with dGTP (FIG. 5A control lane) corresponding to the guanines in the TTAGGG repeat.

Similar results were observed when dATP was replaced by 7-deaza-dATP with 1 mM TTP and 1.56 μ M [α - 32 P]dGTP (FIG. 6A and FIG. 6B). The products were sensitive to RNase A and processivity decreased with increasing 7-deaza-dATP concentrations. No telomerase activity was detected at > 1.5 mM 7-deaza-dATP. However, the level of telomerase activity remained unchanged with 0.25-2.0 mM dATP as a substrate. Unlike what is observed with 7-deaza-dGTP, the presence of 7-deaza-dATP does not result in a change in pause sites compared to the reactions in the presence of dATP.

The present work shows that a pause site can shift to bands two bases smaller in the presence of excess TTP and dGTP and limiting concentrations of [α - 32 P]dATP (FIG. 7A) which would be the second thymine. The telomerase ladder was difficult to obtain

with concentrations less than 3.12 μM of [$\alpha\text{-}^{32}\text{P}$]dATP. In addition, a telomerase ladder was not generated in the presence of limiting radioactive dTTP. The dependence of the location of the pause sites on the concentration of nucleotides has also been demonstrated in *Tetrahymena* (Greider & Blackburn, 1987).

5

J. Inhibitory and Modulating Effect of 7-Deaza Nucleotides

The compounds 7-deaza-dGTP and 7-deaza-dATP have been found to be potent inhibitors and modulators of telomerase activity in the S100 extracts of 293 cells. The level of inhibition of the radioactive native substrate by these compounds is comparable
10 to that caused by the cold native substrate. Even in the presence of high concentrations of the native nucleotide (1-2 mM), the 7-deaza nucleotides are able to inhibit telomerase activity with IC_{50} values of less than 100 μM . These results suggest that both 7-deaza-dGTP and 7-deaza-dATP compete well with the natural substrates, dGTP and dATP.

7-Deaza-dGTP and 7-deaza-dATP can replace the native purine nucleotides,
15 dGTP and dATP respectively. The total activity is weak and shorter ladders are formed when the 7-deaza analogues replace their native nucleotides. In comparison, 7-deaza purine nucleotides are effective substrates for a variety of other DNA polymerases (Mizusawa *et al.*, 1986; McConlogue *et al.*, 1988; Seela & Roling, 1991). There are several possibilities why N7 is a factor in telomerase processive activity.

20 One explanation for the selective effect of 7-deaza nucleotides involves the propensity of G-rich DNA sequences to form secondary structures involving G-quartets. The oligodeoxynucleotide having the sequence d(GGTTAGGGTTAG), corresponding to the human telomeric DNA sequence, forms G-quartet structure, as evidenced by ^1H NMR (FIG. 8). The imino proton spectrum of this sequence
25 demonstrates diagnostic signals in the region of 10-12 ppm indicating G-quartet formation. As the temperature of this sample is raised, these signals disappear as the G-quartet dissociates. In KCE-containing buffer, these signals up to 70 $^{\circ}$ C due to the ability of potassium ions to stabilize G-quartets. N7 is involved in G-quartet and hairpin structures. Replacing dG with dI in various telomeric sequences disrupted G-tetraplex

formation but had no inhibitory effect on telomerase *in vitro* (Henderson *et al.*, 1990). Furthermore, it has been shown that G-quartet structures may actually inhibit the initiation of telomerase activity (possibly by prevention of binding of telomerase to the oligonucleotide). However, it has been proposed that G-tetraplex structures can facilitate the translocation step (Zahler *et al.*, 1991). Incorporation of the 7-deaza nucleotides can prevent formation of these structures. The oligodeoxyribonucleotide having the sequence d(GGTTAGG*GTTAG) when G* represents 2'-deoxy-7-deazaguanosine, in KCL-containing buffer displays only weak and broad imino proton signals in the G-quartet dianostic region of 10-12 ppm in its low temperature ¹H NMR spectrum. These weak signals disappear as the sample is warmed, and are no longer present at 25° C (FIG. 9) making the translocation step more difficult. The result of this would be formation of shorter telomerase products. The telomerase ladder formed in the presence of 7-deaza-dATP is also prematurely shortened. While it is possible that N7 of adenine may also be involved in the formation of stable G-tetraplex structures, there is conflicting evidence to that effect (Murchie & Lilley, 1994; Balagutumoorthy & Brahmachari, 1994). Thus, it remains to be seen if addition of one 7-deaza-2'-deoxyadenine per TTAGGG repeat along the growing chain is sufficient to disrupt G-quartet structures or hairpin formation.

Another explanation for the effect of 7-deaza nucleotides on telomere formation is that incorporation of 7-deaza nucleotides destabilizes the DNA-telomerase RNA duplex or causes a conformational change resulting in complete dissociation of the growing strand from telomerase. It has been shown that replacing dG with 7-deaza-dG in alternating d(G-C) or d(C-G) oligomers does not change the conformation from B DNA but does result in a decrease in melting temperatures (Seela & Driller, 1989). Replacing dA with 7-deaza-dA in homoligomers dA:dT also results in a decrease in stability. However, alternating d(A-T) oligomers in which dA is replaced by 7-deaza-dA are slightly more stable than d(A-T) oligomers. Replacing dA with 7-deaza-dA in homoligomers dA:dT also results in a decrease in stability. However, alternating d(7-deazaA-T) oligomers are slightly more stable than d(A-T) oligomers (Seela & Thomas,

1995). In addition, replacing dA with 7-deaza-dA within poly dA tracts reduces the degree of bending (Seela *et al.*, 1989). Dissociation of the enzyme from the growing strand may account for decrease in processivity of telomerase as the 7-deaza nucleotides are incorporated. Furthermore, a change in stability or conformational change caused by
5 addition of one or more 7-deaza-dG's per TTAGGG repeat may cause telomerase to dissociate during the elongation step. This could be the cause of the shift and increase in number of pause sites observed in the presence of 7-deaza-dGTP but not 7-deaza-dATP.

A third explanation involves substrate inhibition. The substrate inhibition seen at higher 7-deaza-dGTP and 7-deaza-dATP concentrations has been observed for a variety
10 of other enzymes (Dixon & Webb, 1979). Substrate inhibition is also seen in *Tetrahymena* in 10 mM dGTP (and 2.5 μ M [α -³²P]TTP) which is 1,000-10,000 fold greater than the 1-10 μ M dGTP required for optimum activity (Collins & Greider, 1995). Substrate inhibition could be due to two mechanisms (Dixon & Webb, 1979). In the first mechanism, the nucleotides can bind to the telomerase active site to form other
15 ineffective telomerase-nucleotide complexes which inhibit telomerase activity. The less effective binding modes have a higher K_m than the correct mode so that inhibition becomes more apparent only when higher concentrations of substrate is present.

In the second mechanism, the nucleotides can bind to a site other than the active site which causes a conformational change resulting in inhibition. This binding would
20 have a lower affinity than the active site binding so that inhibition becomes more apparent with increasing substrate concentrations. When the 7-deaza nucleotide competes with the radioactive nucleotide at limiting concentrations, 1-3 μ M, the observed IC_{50} value most likely reflects the direct competition between the cold and labeled nucleotide. However, the observed inhibition of telomerase activity by the 7-deaza nucleotides in the
25 presence excess concentrations of the nonradioactive native nucleotide may be due to substrate inhibition. Details of human telomerase structure are unknown at this time; thus, how the substitution of a carbon for N7 is responsible for the observed substrate inhibition is not yet fully defined.

K. Pharmaceutical Compositions

The pharmaceutical compositions disclosed herein may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of the unit. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup of elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

The active compounds may also be administered parenterally or intraperitoneally. Solutions of the active compounds as free base or pharmacologically acceptable salts can

be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

- 5 The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of
- 10 microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of
- 15 dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the
- 20 compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

- Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions
- 25 are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-

drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

For oral prophylaxis the compounds may be incorporated with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an antiseptic wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

The composition can be formulated in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like.

Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

5 Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with
10 sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or
15 injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet
20 sterility, pyrogenicity, general safety and purity standards as required by FDA standards.

* * * * *

The following examples are included to demonstrate preferred embodiments of
25 the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific

embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

MATERIALS AND METHODS

5

Abbreviations used: Tris-HCl, Tris(hydroxymethyl)-aminomethane hydrochloride; MEM, minimal essential medium; HEPES, N-[2-Hydroxyethyl]piperazine-N'-[2-ethansulfonic acid]; PMSF, Phenylmethylsulfonic acid; EDTA, ethylenediaminetetraacetate; dGTP, 2'-deoxyguanosine-5'-triphosphate; dATP, 2'-deoxyadenosine-5'-triphosphate; TTP, 2'-deoxythymidine-5'-triphosphate; dG, 2'-deoxyguanosine; dA, 2'-deoxyadenosine; dC, 2'-deoxycytidine

Materials

Oligonucleotide primers (Genosys), dNTP's including 7-deaza-dGTP and 7-deaza-dATP (Pharmacia), [α -³²P]dGTP and [α -³²P]dATP (Dupont NEN), RNase A, PMSF, pepstatin A, and leupeptin, and other chemicals (Sigma).

Methods

1. Telomerase Preparation

Telomerase was prepared from transformed human embryonic kidney 293 cell line. Human embryonic kidney 293 cells were grown in Joklik's modified MEM medium (Sigma, St Louis, MO) supplemented with 5% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO₂. The cells were subcultured at 1-2 x 10⁵ cells/ml twice a week. When the cells grew to approximately 1 x 10⁶ cells/ml, the cells (~2 x 10⁹ cells) were centrifuged and transferred into polypropylene centrifuge (50 ml) tubes and kept on ice. The cells were then centrifuged in a (Beckman GS-6R) centrifuge at 1200 RPM for 10 minutes at 4°C, and resuspended in PBS. The washed cells were resuspended in 15 ml Hypobuffer Mix (10 mM HEPES, pH 8.0, 3 mM KCl, 1 mM MgCl₂, 1 mM

dithiothreitol, 1 μ M leupeptin, 0.1 mM PMSF, 10 μ M pepstatin, 0.83 μ g/ml chymostatin, 0.83 μ g/ml antipain, and 43.2 units/ml RNase Guard) and centrifuged. The cell pellets were resuspended in 0.75 x volumes Hypobuffer [Hypobuffer Mix + 40 units/ml RNase Guard (Pharmacia, NJ) to allow the cells to swell. The cell suspensions were
5 homogenized to allow the telomerase to "leak" into the cytosolic fraction. The lysate was then poured into new, autoclaved, 3 ml polyallomer ultracentrifuge tubes and centrifuged at 100,000 x g for 1 hour at 4°C. A top lipid layer was carefully removed and discarded, and the supernatant was collected. 1/50 of the volume of 5M NaCl was added to the clear
10 supernatant fluid to achieve a final concentration of 0.1 M. Sterile glycerol was added until the final glycerol concentration was 20%. The enzyme solution was aliquoted into Eppendorf tubes and quickly frozen in liquid nitrogen and stored at -70°C.

2. Conventional Telomerase Assay (32 P-dGTP as Radiolabel Substrate)

The assay is based on the ability of the telomerase enzyme in the S100 fractions to
15 recognize an 18-base DNA primer with the DNA sequence (TTAGGG)₃. DNA polymerization initiates from the 3'-OH group of the oligonucleotide. By reading from its complementary internal RNA template, telomerase adds a repeated set of 5'-TTAGGG-3' to the original 18-base primer. Non-synchronous "starts" and "terminations" among the different enzyme complexes result in a degenerative set of
20 different length DNA primers occurring in 6-base increments. Incorporation of [α - 32 P]dGTP at the guanine positions of the 5'-TTAGGG-3' sequence allows the newly synthesized DNA fragments to be visualized by autoradiography. The resulting image forms the characteristic "ladder" structure that is used to identify telomerase activity. The images are scanned using a phosphorimager or laser densitometer (Molecular Dynamics,
25 Sunnyvale, CA.). Comparisons of the total volumes under the corresponding peaks are used to quantitate the total amount of [α - 32 P] incorporated in the primer extension reaction.

The telomerase assay was initiated by mixing 20 μ l of 2x reaction mix (100 mM Tris-OAc, pH 8.5, 100 mM KOAc, 10 mM β -mercaptoethanol, 2 mM spermidine, 2 mM MgCl_2 , 2 mM dATP, 2 mM dTTP, 2 μ M oligo primer, potential inhibitors of interest at the appropriate concentration, and 50 μ Ci [α - 32 P]dGTP) with 20 μ l of S100 enzyme
5 extract. The reaction mixture was then incubated at 30°C for 1 hour. The reaction was stopped by incubating sequentially with 50 μ l of the stop solution (10 mM Tris pH 7.5, 20 mM EDTA, 0.1 mg/ml RNase A) and 50 μ l of deproteinase solution (10 mM Tris pH 7.5, 0.5% SDS, 0.3 mg/ml Proteinase K). The mixture was extracted with 50% phenol/50% chloroform (v/v). The DNA was then precipitated by adding 2.5x volumes
10 of cold EtOH and letting it stand overnight at -70°C. The DNAs were separated on an 8% polyacrylamide/7M urea denaturing gel at 1600 V for 1.5 hours. The gel was dried and exposed to Kodak XAR-5 film to gel in -70°C. Typical autoradiographic exposures required 3-14 days.

15 3. Modified Telomerase Assay (^{32}P -dATP as Radiolabeled Substrate)

In the conventional telomerase assay, the concentrations of both non-radiolabeled nucleotides, dATP and dTTP, are 1 mM, and the concentration of the radiolabeled [^{32}P]dGTP is 1.56 μ M. Because of the high concentration of dATP and dTTP, the standard assay may not be suitable for determining the potential telomerase inhibition of
20 any "Adenine or Thymine" analogs. A modified alternative telomerase assay was developed which uses dGTP (1 mM) and dTTP (1 mM) as the non-radiolabeled substrates and 3.12 μ M [α - ^{32}P]-dATP as the radiolabeled substrate. All other chemicals and reaction conditions are identical to the conventional assay.

25 4. Testing Selected Nucleoside Triphosphates as Telomerase Substrates

As described in Section 2, telomerase catalyzes the incorporation of dATP, dTTP and [α - ^{32}P]dGTP into a (TTAGGG)₃ primer. In order to determine whether dATP analog can be incorporated into telomere, native dATP in the standard telomerase reaction is replaced with various concentrations of dATP analogs. Following the reaction,

telomeric products are isolated and analyzed as described in Section 2. Similarly, native dTTP in the standard telomerase reaction can be replaced by a structurally modified dTTP analog to test whether the dTTP analog can be used as a telomerase substrate.

In order to evaluate whether or not a dGTP analog can be incorporated by telomerase, native dGTP in the modified telomerase reaction (1 mM dGTP, 1 mM dTTP and 3.12 μ M [α^{32} P]dATP) is replaced with the dGTP analog.

5. Testing Selected Nucleotides for Their ability to Destabilize of G-quartet Formation.

The oligodeoxyribonucleotide having the sequence d(GGTTAGGGTTAG), corresponding to the human telomeric DNA sequence, was prepared using standard automated solid phase synthesis techniques employing a ten micromole synthesis on an Applied Biosystems Model 381A automated DNA synthesizer. Solid support and reagents were purchased from Biogenex, Glen Research, or Applied Biosystems, South San Francisco, CA. The DNA sample was deprotected in concentrated NH_4OH at 55°C overnight, purified by reverse phase HPLC on a C18 column (Dynamax-300A) and then dialyzed extensively against deionized water. NMR samples were prepared by reconstituting lyophilized DNA in 90% H_2O /10% D_2O containing 1 mM EDTA/50 mM potassium phosphate and either 100 mM KCl or 100 mM NaCl. NMR experiments were carried out on a Bruker AMX 500 MHz spectrophotometer using a 1-1 echo pulse sequence with maximum excitation centered as 12.0 ppm. At temperatures as high as 70°C, the NMR spectrum of the sample dissolved in KCl buffer displayed characteristic resonances in the region of 10-12 ppm that corresponded to the imino protons of the G-quartet structure. The same sample dissolved in NaCl buffer only displayed these imino resonances at 10-12 ppm up to temperatures of 60°C, indicating that these resonances correspond to the potassium-stabilized G-quartet structure.

In a similar manner, the fully protected phosphoramidite of a selected nucleotide analog was used in an automated solid phase DNA synthesizer to synthesize a oligodeoxyribonucleotide having the same sequence but with one natural base substituted

with the nucleotide analog, for example d(GGTTAGG*GTTAG) where G* represents a 7-deaza-2'-deoxyguanosine residue. After cleavage from the solid support, deprotection and purification, the oligodeoxyribonucleotide is dissolved in 90% H₂O/10% D₂O containing 100 mM KCl/1 mM EDTA/50 mM potassium phosphate. The imino NMR spectrum of this oligodeoxyribonucleotide is acquired at various temperatures from about 0°C-80°C and the presence of the diagnostic G-quartet imino is indicated by the presence of resonances at 10-12 ppm.

6. Growth Inhibition Studies

Exponentially growing cells ($1-2 \times 10^3$ cells, unless specified otherwise) in 0.1 ml medium were seeded on day 0 in a 96-well microtiter plate. On day 1, 0.1 ml aliquots of medium containing graded concentrations of test analogs were added in duplicate to the cell plates. After incubation at 37°C in a humidified incubator for 6 days, the plates were centrifuged briefly and 100 µl of the growth medium was removed. Cell cultures were incubated with 50 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, (MTT) at a concentration of 1 mg/ml in Dulbecco's phosphate buffered saline (PBS)) for 4 hr at 37°C. The resulting purple formazan precipitate was solubilized with 200 µl of 0.04 N HCl in isopropyl alcohol. Absorbance was monitored in a BioRad Model 3550 Microplate Reader at a test wavelength of 570 nm and a reference wavelength of 630 nm. The absorbance was transferred to a PC 486 computer. The IC₅₀ values were determined by a computer program (EZ-ED50) that fits all the data to the following four-parameter logistic equation:

$$Y = \frac{A_{max} - A_{min}}{1 + (X/IC_{50})^n} + A_{min}$$

where A_{max} is the absorbance of control cells, A_{min} is the absorbance of cells in the presence of highest agent concentration, Y is the observed absorbance, X is the agent

concentration, IC_{50} is the concentration of agent that inhibits the cell growth by 50% of control cells (based on the absorbance) and n is the slope of the curve.

7. Detection of AZTTP Formation in Cells

5 Tumor cells were incubated with graded concentrations of [3H]AZT at 37°C. At the indicated intervals, 2×10^7 cells were centrifuged at 1000 x g for 6 min and were washed once with 5 ml of cold 0.9% NaCl. Cold perchloric acid (4%; 0.45 ml) was added to the pellets, and the tubes were vortexed vigorously. The cold perchloric acid mixture was placed in ice for 20 min and was vortexed occasionally. The precipitated
10 protein and nucleic acids were removed by centrifugation at 3000 x g for 20 min at 4°C. The supernatant fluids (4°C) were then neutralized to pH 6.5 to 7.5 by 5 N KOH, the precipitated potassium perchlorate was removed by centrifugation, and the supernatant fluids were frozen for HPLC analysis. HPLC analysis was performed on a Beckman HPLC. The separation was achieved by a linear gradient elution of potassium phosphate
15 buffer pH 4.5 (10 to 500 mM) on a Whatman strong anion exchange column, Partisil 10 SAX.

8. Determination of the Effect of Agents on Telomere Length

HeLa cells were cultured in DMEM medium supplemented with 10% fetal bovine
20 serum in the presence of testing agents (at the indicated concentration $\sim IC_{10}$ value). The cells were subcultured every 3.5 days. The cells were grown for 4 passages which is equivalent to ~ 12 cell population doublings. At the appropriate cell passage, $\sim 1 \times 10^7$ HeLa cells were harvested by trypsin treatment. High quality DNA (large in size) was prepared by Proteinase K and RNase treatment, followed by phenol extraction. DNA
25 isolated from the control and analog-treated HeLa cells was digested with restriction enzyme *MseI* to completion. Restriction digested DNAs were electrophoresed on a 0.8% agarose gel. The gel was Southern blotted onto a nitrocellulose or nylon membrane and the membrane was probed by hybridization with a telomere specific sequence containing TTAGGG repeats. The membrane was exposed to autoradiographic film. After

development, the signals in each lane were scanned and quantitated by video (CCD) densitometry. The median length, peak, and other curve characteristics were determined by computer (using Image 1.5). Duplicate samples were analyzed for each compound.

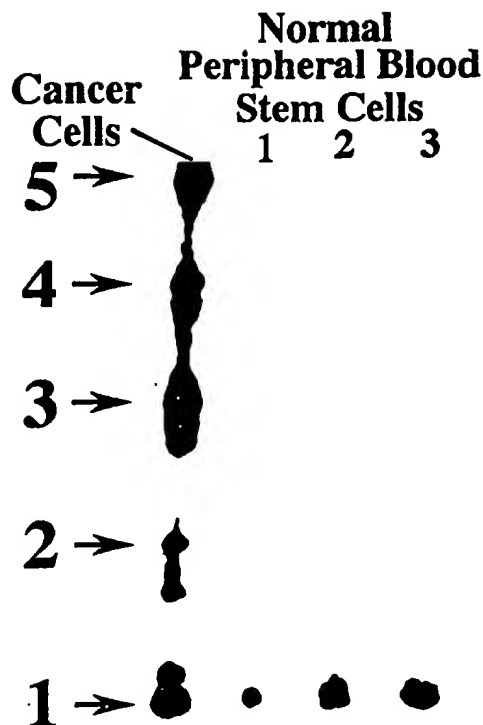
5 **9. Induction of Dicentric Chromosome by a Telomerase Inhibitor**

The same cells grown for the telomere analysis were plated for chromosome preparation. Metaphase cells were harvested, fixed by methanol/ acetic acid and the chromosomes spread on slides for fluorescent *in situ* hybridization analysis. The spreads were hybridized with a centromere probe and detected by a fluorescein tag. The number
10 of fused chromosomes per cell was calculated for each compound tested.

EXAMPLE 1

15 **A. Normal Cells Produce Non-Processive Telomerase While Cancer Cells
Produce Processive Telomerase**

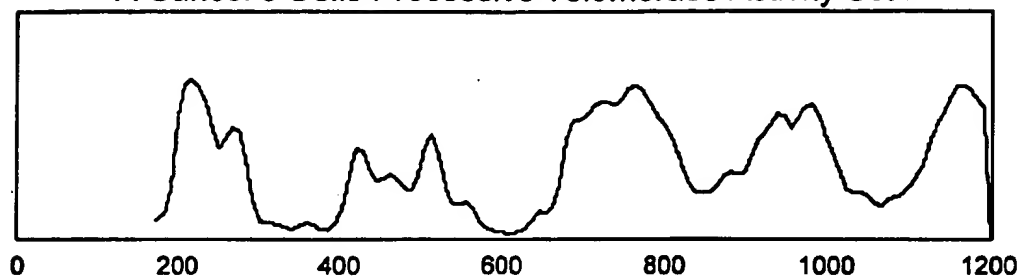
Telomerase was prepared from various human cancer cells and normal stem cells. Telomerase activity was compared using the conventional telomerase assay (see section 2 under Materials and Methods) and modified conventional telomerase assay see section 3 under Materials and Methods). Telomerase incorporates labeled dGTP into a telomeric
20 primer by adding a telomeric repeat or repeats. The labeled primers were electrophoresed and visualized by autoradiography. Telomerase activity is indicated below in the first lane on the left is from human renal cell carcinoma cancer cells. The telomerase product bands are indicated by numbered arrows. This activity is processive as indicated by the multiple bands produced by the cancer telomerase adding multiple telomeric repeats. The
25 telomerase activities in lane marked 1,2, and 3 are from normal human blood stem cells. These normal telomerase activities are non-processive as indicated by the single band produced by normal telomerase adding a single repeat to the primer.



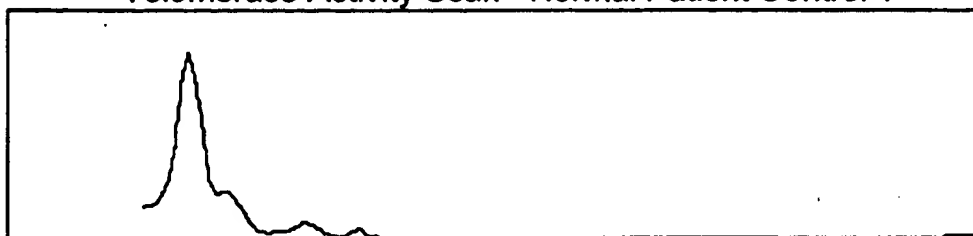
B. Densitometric Scans of the Telomerase Activities from Cancer Cells and Normal Stem Cells

- 5 The density of signal in the lanes shown in the activity scan shown below was determined along each lane. The scans show in a quantitative manner that cancer cell telomerase activity is processive and distinctly different from the non-processive telomerase activity of normal cells. The numerous peaks past coordinate position 300 are indicative of processive telomerase activity. Normal cells essentially have no peaks past
- 10 position 300 and only have the first peak, which is indicative of non-processive telomerase activity.

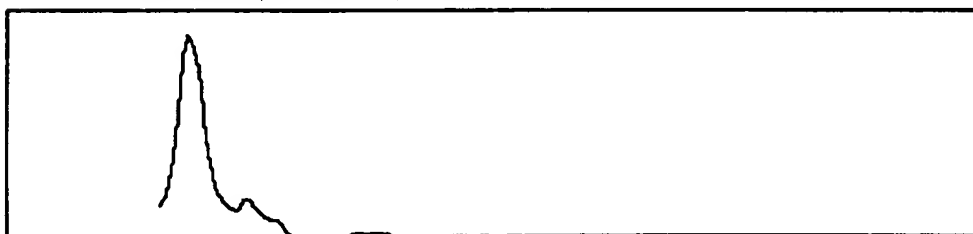
A Cancer's Cells Processive Telomerase Activity Scan



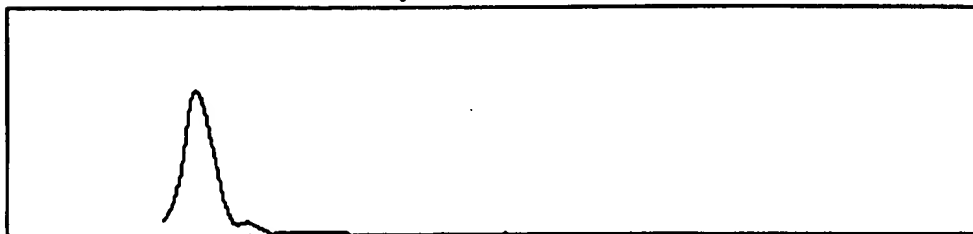
**Normal Peripheral Blood Stem Cell's Non-Processive
Telomerase Activity Scan - Normal Patient Control 1**



**Normal Peripheral Blood Stem Cell's Non-Processive
Telomerase Activity Scan - Normal Patient Control 2**

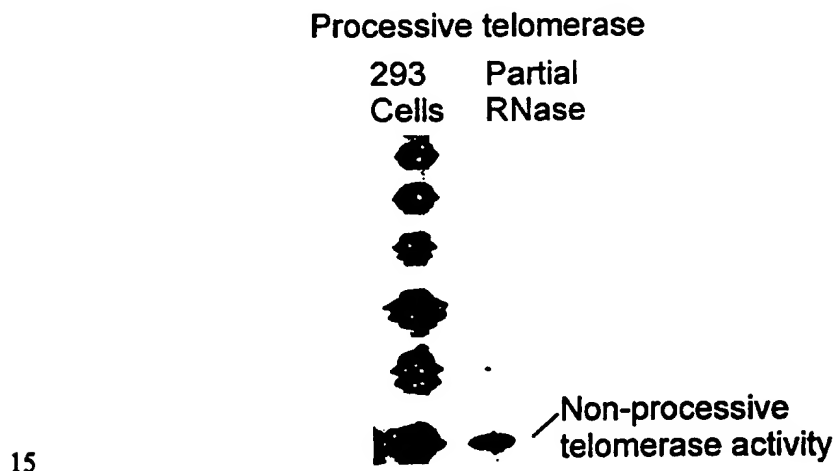


**Normal Peripheral Blood Stem Cell's Non-Processive
Telomerase Activity Scan - Normal Patient Control 3**



C. Conversion of Processive Telomerase to Non-processive Telomerase

Human cancer telomerase was incubated with limited amounts of RNase A, and then assayed using the conventional telomerase assay where labeled dGTP is incorporated into the telomerase products. The products were separated by electrophoresis, and visualized by autoradiography. The left lane of autoradiogram below shows the human cancer telomerase activity without treatment and the right lane shows the same telomerase activity with RNase treatment. The limited amount of RNase partially cleaved the telomerase RNA component so that the telomerase acts non-processively. This is consistent with the RNA component having structures required for the processive function of telomerase. These structures can be targeted to convert a cancer's aggressive processive telomerase activity to a non-aggressive or benign and non-processive activity.



D. Modulation of Human Telomerase Exonuclease to Promote Shortening of Telomeres

Human processive telomerase exhibits a 3' to 5' exonuclease activity that can be stimulated to remove telomeric sequence rather than synthesize telomerase sequence.

Human cancer telomerase was incubated in the presence of a limited amount of dTTP. The autoradiogram below shows the exonuclease activity of telomerase where a reduction in nucleotides such as dTTP and a change in the DNA primer substrate can induce the exonuclease activity. As the exonuclease removes G residues from the primer, the polymerase of telomerase can fill them back in, thus labeling the original primer with labeled dGTP.



Telomerase activity was determined by the conventional assay using an 18-mer telomeric sequence primer, and the products were separated by electrophoresis. The two left lanes show products revealing that telomerase has removed a significant amount of the telomeric sequence as the result of a 3' to 5' exonuclease. This activity was stimulated by the limitation of dTTP (e.g. 100mm) and is not normally observed under normal conditions (e.g. 1mm).

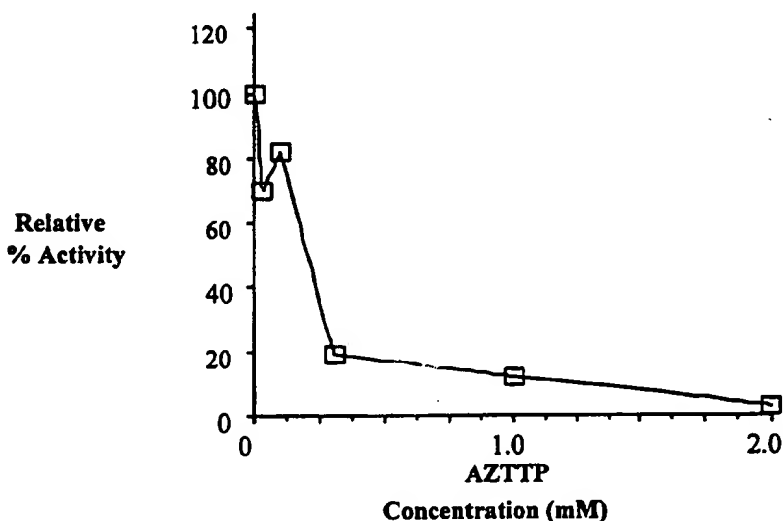
EXAMPLE 2.

Inhibition of human cancer 293 cell telomerase activity by selected thymidine triphosphates.

The following examples demonstrate that thymidine triphosphates with modifications on the 2'-deoxyribose moiety or on the heterocyclic moiety are capable of inhibiting human telomerase activity. The studies were performed using the conventional telomerase assay in which the competing dTTP concentration is in the range of 1-2 mM.

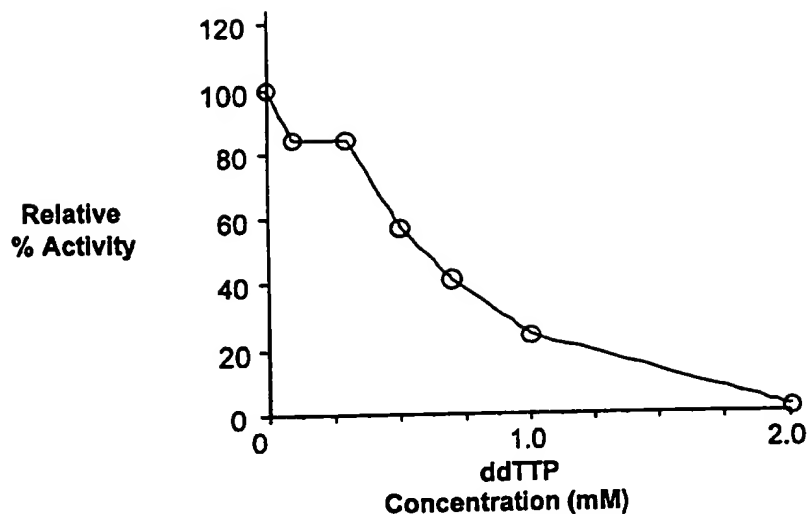
A. Inhibition of Human Cancer Telomerase by 3'-Azido-3'-deoxythymidine-5'-triphosphate (AZTTP, NA010).

Telomerase was prepared from 293 cells. The conventional telomerase assay was performed where telomerase incorporates labeled dGTP into a telomeric primer and the labeled primers are electrophoresed and signal quantitated by autoradiography and densitometry. Telomerase was incubated with concentrations of AZTTP ranging from 0 to 2 mM. The IC_{50} was ~200 μ M. The relative percent activity of AZTTP at different concentrations is shown below.



B. Inhibition of Human Cancer Telomerase by 2',3'-Dideoxythymidine-5'-triphosphate (ddTTP, NA001).

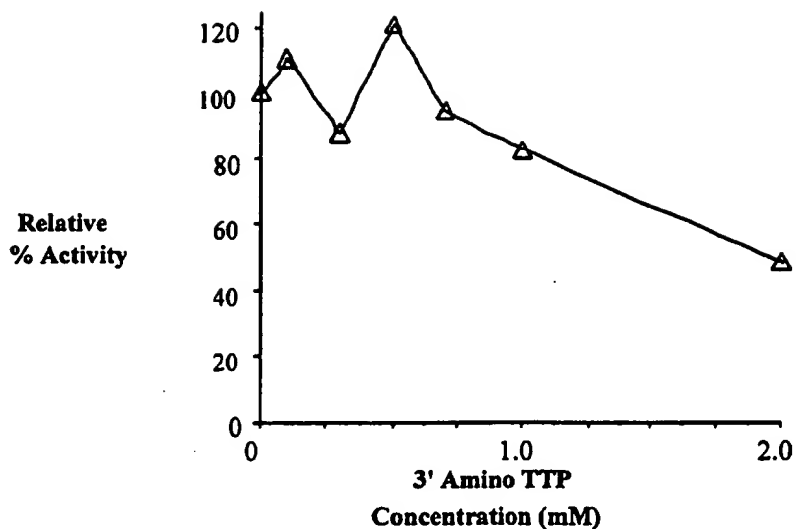
Telomerase was prepared from 293 cells. The conventional telomerase assay was performed where telomerase incorporates labeled dGTP into a telomeric primer and the labeled primers are electrophoresed and signal quantitated by autoradiography and densitometry. Telomerase was incubated with concentrations of ddTTP ranging from 0 to 2 mM. The IC_{50} was ~500 μ M. The relative percent activity of the ddTTP with increasing concentration is shown below.



C. Inhibition of Human Cancer Telomerase by 3'-Amino-2'-deoxythymidine-5'-triphosphate (3'-Amino-TTP, NA005).

Telomerase was prepared from 293 cells. The conventional telomerase assay was performed where telomerase incorporates labeled dGTP into a telomeric primer and the labeled primers were electrophoresed and signal quantitated by autoradiography and densitometry. Relative activity change with concentration is shown in the graph below.

Telomerase was incubated with concentrations of 3'-Amino-TTP ranging from 0 to 2 mM. The IC_{50} was ~2 mM.



5 **D. Inhibition of Human Cancer Telomerase by 5-Allylamino-2'-deoxythymidine-5'-triphosphate (NA021).**

NA021 is an analog of dTTP in which the methyl group is replaced with an allylamino group. NA021 demonstrated a concentration dependent inhibition of 293 cell telomerase with an estimated IC₅₀ value of ~0.5 mM. This is shown in Table2.

10

TABLE 2

NA021(mM)	% of Control
10	100
0.125	51
0.25	70
0.5	43
1.0	33
2.0	30

15

20 **EXAMPLE 3**

Inhibition of human cancer 293 cell telomerase activity by selected deoxyguanosine triphosphates.

The following examples demonstrate that 2'-deoxyguanosine triphosphates with modifications on the phosphate moiety, on the sugar moiety or on the heterocyclic moiety are capable of inhibiting human telomerase activity. Since the testing analogs are dGTP analogs, the inhibitory activity was evaluated using the conventional telomerase assay.

5

A. Lack of Telomerase Inhibitory Activity by Guanosine-5'- β,γ -methylene triphosphate (NA011).

Compound NA011 - (a guanine ribonucleotide and not a 2'-deoxyribonucleotide) was inactive as telomerase inhibitor. Compared with the results for compound NA014, 2'-deoxyguanosine-5'- β,γ -methylene triphosphate (see Example 3. B), the 2'-deoxyribose appears to be required for inhibitory activity.

TABLE 3A

	NA011(mM)	% of Control
15	0.125	82
	0.25	63
	0.5	86
	1.0	70
	2.0	65

20

B. Inhibition of Human Cancer Telomerase Activity by 2'-Deoxyguanosine-5'- β,γ -methylene triphosphate (NA014)

NA014 is a dGTP analog with a modification on the phosphate moiety in which the oxygen atom linking the β and γ phosphate groups is replaced with a methylene group. Using the conventional telomerase assay, NA014 demonstrated a concentration-dependent inhibition of human 293 cells telomerase activity.

25

TABLE 3B

	NA014(mM)	% of Control
30	0.	100
	0.125	60
	0.25	38
	0.5	42

1.0	40
2.0	28

5 **C. Inhibition of Human Cancer Telomerase Activity by 2'-Deoxyguanosine-5'-O-(1-thiotriphosphate) (NA013)**

NA013 is a dGTP analog with a modification on the phosphate moiety in which the α (P=O) bond is replaced with a P=S bond. Using the conventional telomerase assay, NA013 demonstrated a concentration-dependent inhibition of human 293 cell telomerase activity.

TABLE 3C

	<u>NA013(mM)</u>	<u>% of Control</u>
	0.	100
	0.125	75
15	0.25	47
	0.5	0
	1.0	0
	2.0	0

20

D. Inhibition of Human Cancer Telomerase Activity by 2',3'-dideoxyguanosine-5'-triphosphate (NA020).

NA020 is a dGTP analog in which the 2'-deoxyribose moiety is replaced with a 2',3'-dideoxyribose moiety. Using the conventional telomerase assay, NA020 completely at concentrations as low as 12.5 μ M (0.0125mM) inhibits human 293 cell telomerase activity.

TABLE 3D

	<u>NA020(mM)</u>	<u>% of Control</u>
	0.	100
30	0.0125	0
	0.025	0
	0.05	0
	0.1	0
	0.2	0
35	0.5	0
	1.0	0

E. Inhibition of Human Cancer Telomerase Activity by 7-Deaza-2'-deoxyguanosine-5'-triphosphate (NA022).

NA022 is an example of a dGTP analog in which the heterocyclic base, guanine, is replaced with a 7-deazaguanine. Using both the conventional telomerase assay and the modified assay, NA022 demonstrated concentration-dependent inhibition of human 293 cell telomerase activity.

TABLE 3E

	<u>NA022(μM)</u>	<u>% of Control</u>
10	0.	100
	1.0	99
	2.5	114
	5.0	67
	7.5	81
15	10	24
	25	27
	50	18
	75	0
	100	0

EXAMPLE 4.

Inhibition of Human Cancer 293 Cell Telomerase Activity by Selected Deoxyadenosine Triphosphates

The following examples demonstrate that 2'-deoxyadenosine triphosphates with modifications on the phosphate moiety, on the sugar moiety or on the heterocyclic moiety are capable of inhibiting human telomerase activity. Both conventional and modified telomerase assays were used to determine the inhibitory activity of selected deoxyadenosine triphosphate analogs.

A.i. Lack of Inhibition of Human Cancer Telomerase Activity by 2'-Deoxyadenosine-5'- β,γ -methylene triphosphate (NA004) Using the Conventional Telomerase Assay.

NA004 is a dATP analog with a modification on the phosphate moiety in which the oxygen atom linking the β and γ phosphate groups is replaced with a methylene group. Using the conventional telomerase assay, NA004 demonstrated no inhibition of human 293 cells telomerase activity un to 2 mM.

TABLE 4Ai

NA004(mM)	% of Control
0.	100
0.125	70
0.25	72
0.5	88
1.0	98
2	99

A.ii. Inhibition of Human Cancer Telomerase Activity by 2'-Deoxyadenosine-5'- β,γ -methylene triphosphate (NA004) Using the Modified Telomerase Assay.

On the other hand, NA004 demonstrated a concentration-dependent inhibition of human 293 cell telomerase activity using the modified telomerase assay in which the competing radioactive nucleotides is 3.12 μ M [α^{32} P]dATP.

TABLE 4Aii

NA004(mM)	% of Control
0.	100
0.125	6
0.25	1.7
0.5	0
1.0	0
2	0

B.i. Lack of Inhibition of Human Cancer Telomerase Activity by 2'-Deoxyadenosine-5'-O-(1-thiotriphosphate) (NA006) Using the Conventional Telomerase Assay.

NA006 is an example of a dATP analog with a modification on the phosphate moiety in which the α (P=O) bond is replaced with a P=S bond. Using the conventional telomerase assay, NA006 demonstrated no inhibition of human 293 cells telomerase activity up to 2 mM.

TABLE 4Bi

NA006(mM)	% of Control
0	0
0.125	120
0.25	103
0.5	103
1.0	100
2	113

B.ii. Inhibition of Human Cancer Telomerase Activity by 2'-Deoxyadenosine-5'-O-(1-thiotriphosphate) (NA006) Using the Modified Telomerase Assay.

On the other hand, NA006 demonstrates a concentration-dependent inhibition of human 293 cell telomerase activity using the modified telomerase assay in which the competing radioactive nucleotides is 3.12 μ M [α^{32} P]dATP.

TABLE 4Bii

NA006(mM)	% of Control
0	100
0.125	0
0.25	0
0.5	0
1.0	0
2	0

C.i. Lack of Inhibition of Human Cancer Telomerase Activity by 2',3'-dideoxyadenosine-5'-triphosphate (NA007) Using the Conventional Telomerase Assay.

NA007 is an example of a dATP analog in which the 2'-deoxyribose moiety is replaced with a 2',3'-dideoxyribose moiety. Using the conventional telomerase assay, NA007 demonstrated no inhibition of human 293 cells telomerase activity up to 2 mM.

TABLE 4Ci

	NA007(mM)	% of Control
	0	100
5	0.125	118
	0.25	116
	0.5	141
	1.0	126
10	2	133

C.ii. Inhibition of Human Cancer Telomerase Activity by 2',3'-dideoxyadenosine-5'-triphosphate (NA007) Using the Modified Telomerase Assay.

15 On the other hand, NA006 demonstrated a concentration-dependent inhibition of human 293 cell telomerase activity using the modified telomerase assay in which the competing radioactive nucleotides is 3.12 μ M [α ³²P]dATP.

TABLE 4Cii

	NA007(mM)	% of Control
	0	100
20	0.125	0
	0.25	0
	0.5	0
25	1.0	0
	2	0

D. Inhibition of Human Cancer Telomerase Activity by 7-Deaza-2'-deoxyadenosine-5'-triphosphate (NA023) using both the Conventional and Modified Telomerase Assays.

30 NA023 is an example of a dATP analog in which the heterocyclic base, adenine, was replaced with a 7-deazaadenine. Using both the conventional telomerase assay and the modified assay, NA023 demonstrated concentration-dependent inhibition of human 293 cell telomerase activity.

TABLE 4D

	NA023(μ M)	% of Control
	0	100
5	1.0	69
	2.5	46
	5.0	57
	7.5	40
	10	23
10	25	4
	50	0
	75	0
	100	0

EXAMPLE 5.

Use of Selected Nucleoside Triphosphates as Telomerase Substrate

Human cancer telomerase catalyzes the polymerization of a (TTAGGG)₃ primer using dTTP, dATP, dGTP. Using either the conventional or modified telomerase assay, the following examples illustrate that selected nucleoside triphosphate analogs can replace the native nucleotides (dTTP, dATP, and dGTP). In some examples, incorporation of these nucleotide analogs leads to a prematurely shorten telomere. These examples demonstrate a novel method of affecting telomerase activity.

A. Incorporation of 2'-Deoxyadenosine-5'- β,γ -methylene triphosphate (NA004) by Human Cancer Telomerase

NA004, a dATP analog with a 5'- β,γ -methylene group, is able to replace dATP and become incorporated into telomeric ladders. Using the conventional telomerase assay, NA004 was shown to be incorporated at all the concentrations tested (0.125 - 2 mM), see FIG. 10.

B. Incorporation of 2'-Deoxyadenosine-5'-O-(1-Thio-triphosphate) (NA006) by Human Cancer Telomerase.

NA006, a dATP analog having a P=S bond rather than the P=O bond at the α phosphate group will replace dATP and become incorporated into telomeric ladders. Using the conventional telomerase assay, NA006 was shown to be incorporated at the concentrations tested (0.125 - 2 mM), see FIG. 11.

5

C. Incorporation of 2',3'-dideoxyadenosine-5'-Triphosphate (NA007) by Human Cancer Telomerase

Using the conventional telomerase assay NA007, a dATP analog lacking the 3'-OH group, showed no apparent NA007 incorporation was detected at the concentrations tested (0.125 - 2 mM), see FIG. 12.

10

D. Incorporation of 2'-Deoxyguanosine-5'-O-(1-Thio-triphosphate) (NA013) by Human Cancer Telomerase

NA013, a dGTP analog having a P=S bond rather than the P=O bond at the α phosphate group replaced dGTP and was incorporated into telomeric ladders. Using the modified telomerase assay, NA013 was shown to be incorporated at concentrations of 0.5 - 2 mM, see FIG. 13.

15

E. Incorporation of 2'-Deoxyguanosine-5'- β,γ -methylene triphosphate (NA014) by Human Cancer Telomerase.

20

NA014, a dGTP analog with a 5'- β,γ -methylene group, replaced dGTP and became incorporated into telomeric ladders. Despite the weak activity, using the modified telomerase assay, NA014 incorporated into telomere could be detected at concentrations of 0.5-2mM, see FIG. 14.

25

F. Lack of Incorporation of 2',3'-dideoxyguanosine-5'-Triphosphate (NA020) by Human Cancer Telomerase.

NA020, a dGTP analog lacking the 3'-OH group, once incorporated into telomere was shown to chain-terminate the reaction. Using the modified telomerase assay, no

apparent NA020 incorporation was detected at all concentrations tested (0.125 - 2 mM), see FIG. 15.

G. Incorporation of 7-Deaza-2'-deoxyadenosine-5'-triphosphate (NA023) by Human Cancer Telomerase

dATP was replaced by 7-deaza-dATP (NA023) with 1 mM TTP and 1.56 μ M [α -³²P]dGTP in the conventional telomerase assay). The products were sensitive to RNase A and processivity decreased with increasing 7-deaza-dATP concentrations. No telomerase activity was detected at > 1.5 mM 7-deaza-dATP. However, the level of telomerase activity remained unchanged with 0.25-2 mM dATP as a substrate. The presence of 7-deaza-dATP did not result in a change in pause sites compared to the reactions in the presence of dATP.

H. Incorporation of 7-Deaza-2'-deoxyguanosine-5'-triphosphate (NA022) by Human Cancer Telomerase

Replacing dGTP with 7-deaza-dGTP (NA022) in a modified telomerase reaction containing 1 mM dTTP and 3.12 μ M [α -³²P]dATP resulted in formation of radiolabeled products that were sensitive to RNase A. This reaction was distinguished from a reaction with the native dGTP. First, the total amount of products produced by 7-deaza-dGTP was much less than that produced with dGTP. Second, telomerase appeared to be less processive with 7-deaza-dGTP. As the concentration of 7-deaza-dGTP increased, the products became increasingly shorter and the total amount of products decreased until there was no activity at > 1.5 mM 7-deaza-dGTP. There was no significant change in activity when dGTP is used as a substrate in the reaction with concentrations varying from 0.25-2 mM. Finally, telomerase paused at significantly more sites in the presence of 7-deaza-dGTP. At a concentration of 0.5 mM 7-deaza-dGTP, there was such a large number of bands pertaining to various pause sites that a predominant pause site could not be determined. When the 7-deaza-dGTP concentration is 0.75-1 mM, a repeated triplet of pause sites was distinguishable.

EXAMPLE 6.**Growth Inhibitory Activity of Selected Nucleoside Analogs**

Examples 1-4 demonstrated various dTTP, dATP, and dGTP analogs useful for
5 modulating human tumor telomerase activity either by inhibiting the enzyme activity or
by incorporating into telomere and as the result of incorporation affecting subsequent
telomere elongation. The following examples demonstrate that these compounds have
growth inhibitory activity.

To demonstrate the growth inhibitory activity, one cannot simply incubate the
10 cells with the phosphates (mono-, di-, or triphosphates) and expect them to transport into
the cells and provide the cells with the triphosphates of nucleoside analogs. The
phosphates are highly negatively charged, and therefore do not effectively transport into
cells. This is due to the extracellular dephosphorylation of the mono-, di-, or
triphosphates by alkaline phosphorylase or 5'-nucleotidase back to the nucleoside. With
15 this in mind, the inventors used only nucleosides rather than nucleoside triphosphates in
these studies. Cultured tumor cells were incubated with selected nucleosides at graded
concentrations for 6 days and the growth inhibition was determined by the standard MTT
assay as described in section 6 in the Materials and Methods section.

20 **A. Growth Inhibition of Selected 7-Deaza-2'-deoxynucleoside against
Transformed Embryonic Kidney 293 Cells.**

The following table shows the growth inhibition of the indicated 7-deaza-2'-
deoxynucleoside compounds against transformed human embryonic kidney cells.

25 **TABLE 5**

Code No.	Chemical Name	IC ₅₀ (μM)
FS004	7-Iodo-7-Deaza-2'-Deoxyadenosine	0.319
FS015	7-Iodo-7-Deaza-8-Aza-2'-Deoxyadenosine	1.884
FS018	7-Bromo-7-Deaza-2'-Deoxyadenosine	1.144

Code No.	Chemical Name	IC50 (μ M)
FS004	7-Iodo-7-Deaza-2'-Deoxyadenosine	0.319
FS015	7-Iodo-7-Deaza-8-Aza-2'-Deoxyadenosine	1.884
FS018	7-Bromo-7-Deaza-2'-Deoxyadenosine	1.144
FS017	7-Chloro-7-Deaza-2'-Deoxyadenosine	3.319
FS005	7-Cyano-7-Deaza-2'-Deoxyadenosine	18.162
FS024	7-Methyl-7-Deaza-2'-Deoxyadenosine	108.555
NA024	7--Deaza-2'-Deoxyadenosine	533.320
FS021	7-Iodo-7-Deaza-2'-Deoxyguanosine	63.445
FS020	7-Bromo-7-Deaza-2'-Deoxyguanosine	180.320
FS019	7-Chloro-7-Deaza-2'-Deoxyguanosine	236.706
FS022	7-Methyl-7-Deaza-2'-Deoxyguanosine	488.850
NA025	7-Deaza-2'-Deoxyguanosine	130.220
NA027	1-Methyl-6-Thio-7-Deaza-2'-Deoxyguanosine	161.000
NA028	6-Thio-7-Deaza-2'-Deoxyguanosine	461.000

B. Growth Inhibition of Selected 7-Deaza-2'-deoxynucleoside against Human Colon Carcinoma HT29 Cells.

The following table shows growth inhibition of the indicated 7-deaza-2'-
 5 deoxynucleoside compounds against human colon carcinoma cells.

TABLE 6

Code No.	Chemical Name	IC50 (μ M)
FS004	7-Iodo-7-Deaza-2'-Deoxyadenosine	1.879
FS015	7-Iodo-7-Deaza-8-Aza-2'-Deoxyadenosine	8.090
FS018	7-Bromo-7-Deaza-2'-Deoxyadenosine	0.963
FS017	7-Chloro-7-Deaza-2'-Deoxyadenosine	15.589
FS005	7-Cyano-7-Deaza-2'-Deoxyadenosine	51.230

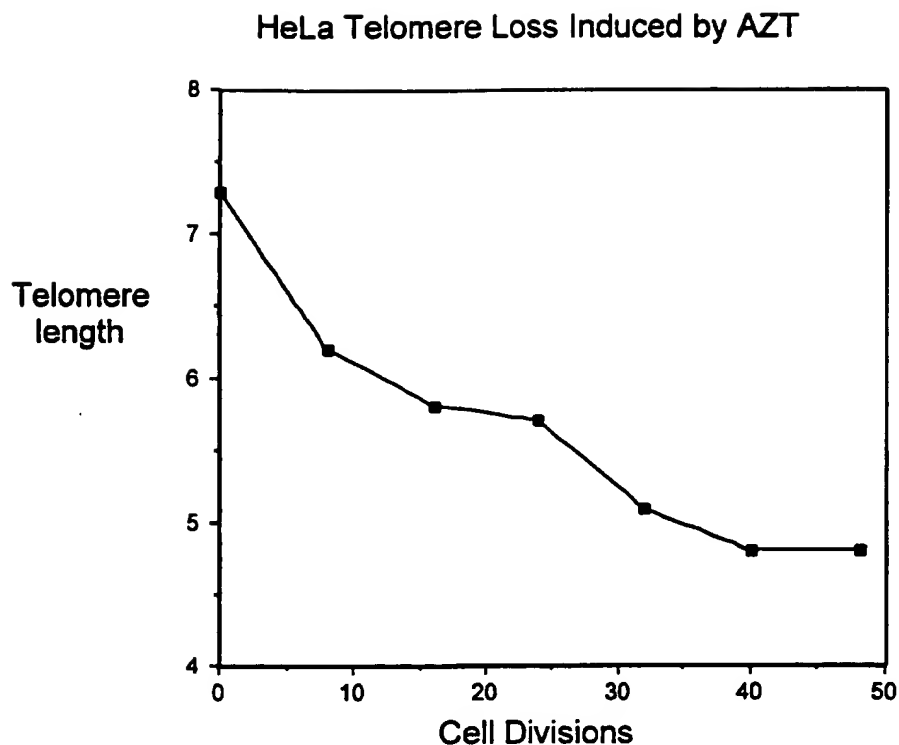
FS024	7-Methyl-7-Deaza-2'-Deoxyadenosine	99.620
NA024	7--Deaza-2'-Deoxyadenosine	146.490
FS021	7-Iodo-7-Deaza-2'-Deoxyguanosine	78.755
FS020	7-Bromo-7-Deaza-2'-Deoxyguanosine	170.310
FS019	7-Chloro-7-Deaza-2'-Deoxyguanosine	108.540
FS022	7-Methyl-7-Deaza-2'-Deoxyguanosine	195.540
NA025	7-Deaza-2'-Deoxyguanosine	101.930
NA027	1-Methyl-6-Thio-7-Deaza-2'-Deoxyguanosine	309.500
NA028	6-Thio-7-Deaza-2'-Deoxyguanosine	461.100

EXAMPLE 7.**Effect of AZT on Cells.**

A reasonable outcome of incubating tumor cells with appropriate 2'deoynucleosides is the formation of the corresponding 2'deoynucleoside triphosphate intracellularly. If 2'-deoxynucleoside triphosphates inhibit intracellular telomerase, one would expect the gradually shortening the telomere length with the formation of dicentric chromosome. Example 6 provided evidence to demonstrate these effects using AZT as the model agent.

A. AZT Induces Telomere Shortening

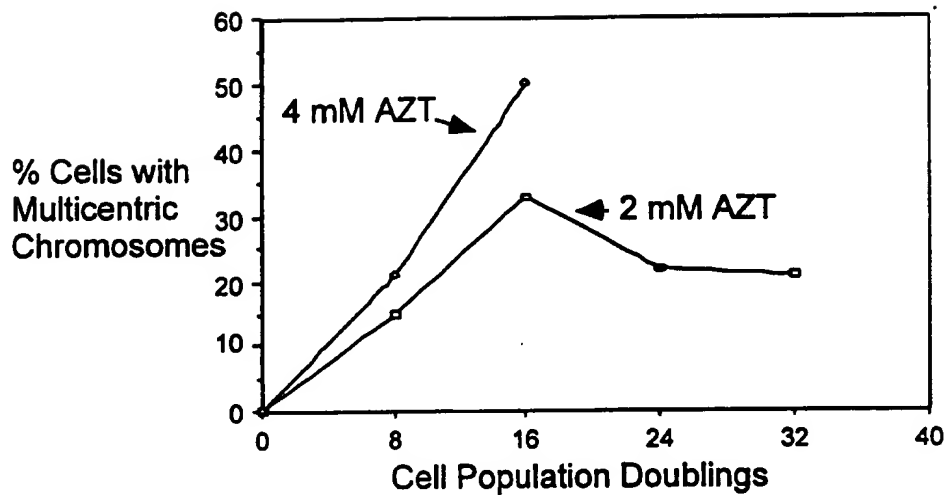
HeLa cells were incubated and grown with 800 μ M of AZT. The amount of AZTTP produced within the cell was expected to inhibit telomerase. Telomeres were measured by digesting the chromosomal ends with *MSEI*. The telomeric fragments were separated by electrophoresis, probed for telomeric sequence and the average size of the signals produced was determined by autoradiography and densitometry. Without telomerase activity, the HeLa cells can no longer maintain their telomeres and therefore the telomeres shorten with each generation. The decrease in telomer length with cell divisions is shown below.



B. AZT Induces Telomere Fusion and Chromosome Instability in CHO Cells.

5 Chinese hamster ovary cells were incubated and grown with 2 and 4 mM AZT. Cytogenetic analysis was performed on the chromosomes and the number of chromosome end fusions was determined. AZT induced a AZT concentration dependent and cell doubling dependent destabilization of the chromosomes by inhibition of telomerase as illustrated in the graph below.

10

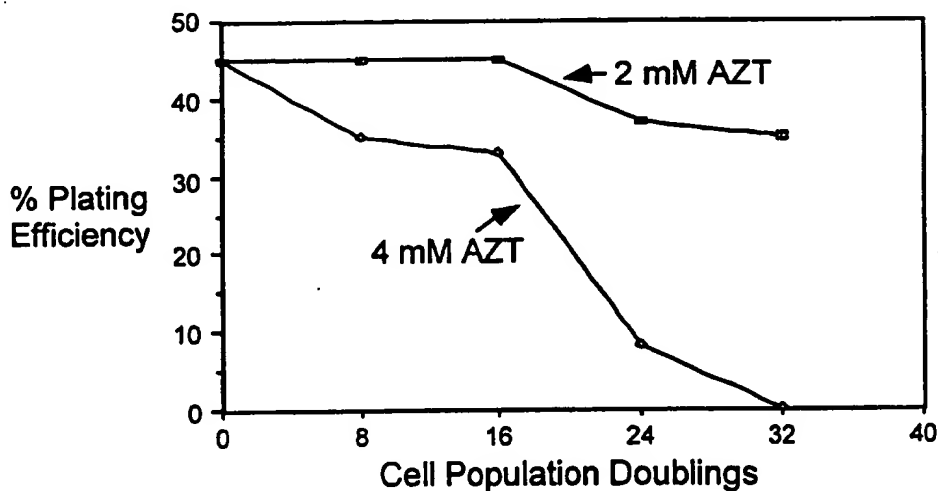


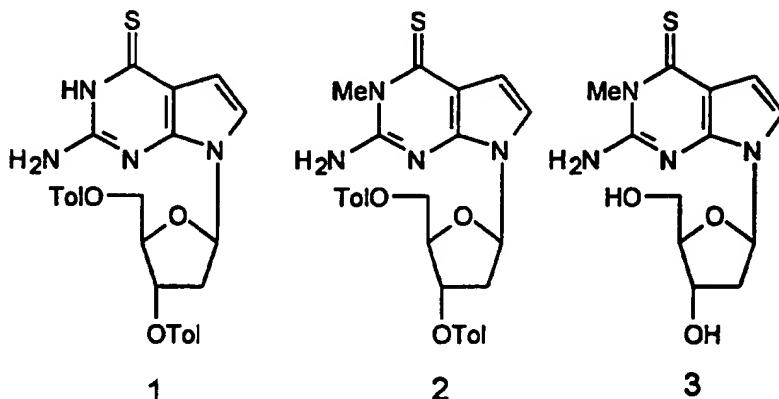
E. AZT Induces Delayed Cell Death in CHO Cells

Chinese hamster ovary cells were incubated and grown with 2 and 4 mM AZT.

- 5 The cell viability was determined by the cells' ability to form colonies. Initially, there was little effect on *viability*, but after 16 cell doublings, the cells began to die in both 2 and 4 mM AZT. Eventually all cells died in 4 mM AZT. Cell death appears to result from massive chromosome instability due to the inhibition telomerase. Results are illustrated below.

10



EXAMPLE 8**Preparation of 7-deaza-1-methyl-6-thio-2'-deoxyguanosine.**

5

1-methyl-2-amino-7-[2'-deoxy-3,5-di-O-(p-toluoxy)]-β-D-erythro-pentofuranose]-7H-pyrrolo[2,3-d]pyrimidine-4-(3H)-thione (2).

To a cooled solution (0 °C, 1 ml DMF) of **1** [Seela, *et al.* (1987)] (300 mg, 0.578 mmol) was added NaH (60% in oil, 25.5 mg, 0.636 mmol). This was allowed to stir for 1 hr at 0 °C. Dimethyl sulfate (73.0 mg, 54.7 μl, 0.578 mmol) was added to the solution at 0 °C. The reaction was stirred for 1 hr and quenched using sat. NH₄Cl. The beige solid was filtered and compound **2** was purified by flash silica gel chromatography (methanol:chloroform 0→5% methanol) to give a white solid (221 mg, 72% yield). R=

15 0.60 (methanol:chloroform 5:95)

1-methyl-2-amino-7-[2'-deoxy-β-D-erythro-pentofuranose]-7H-pyrrolo[2,3-d]pyrimidine-4-(3H)-thione (NA027).

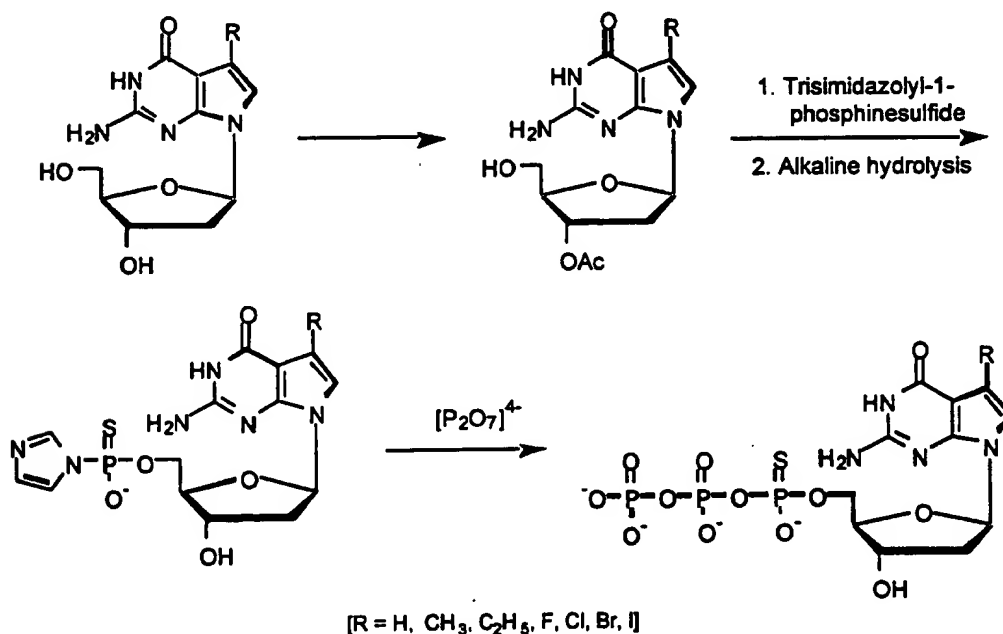
A solution of **2** (180 mg, 0.338 mmol) in 13 ml of 0.1 N sodium methoxide in methanol was stirred at room temperature for 3 hr. The reaction was neutralized with 6 N

20

HCl and evaporated to dryness. The compound, 3, was purified by flash silica gel chromatography (methanol:chloroform 0→10% methanol) to give a white solid (72 mg, 72% yield). R_f 0.27 (methanol:chloroform 10:90). $^1\text{H-NMR}$ (DMSO-d_6) 7.22 (d, 1H, $J=6$ Hz), 6.59 (s, 2H, NH_2), 6.40 (d, 1H, $J=6$ Hz), 6.32 (dd, 1H, $J=6$ Hz and 8 Hz, H-1'), 4.30 (m, 1H, H-3'), 3.78 (m, 1H, H-4'), 3.49 (m, 2H, H-5'), 2.60 (s, 3H, CH_3N), 2.35 and 2.12 (m, 2H, H-2').

EXAMPLE 9

A general procedure for the synthesis of 7-substituted 7-deaza-dGTP α -phosphorothionates is illustrated in the scheme below. dGTP is shown in the scheme but it is equally applicable to the preparation of dATP analogs.



EXAMPLE 10

Destabilization of the ability of human DNA to adopt a G-quartet structure as a result of incorporation of selected 7-deazapurine-2'-deoxyribonucleotides into human telomeric DNA.

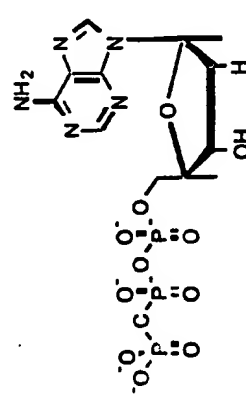
- 5 The incorporation of 7-deaza-2'-deoxyguanosine into an oligodeoxyribonucleotide of the sequence d(GGTTAGG*GTTAG) where G* represents the 7-deaza-2'-deoxyguanosine residue results in the inability of this sequence to form G-quartet structure in KCl-containing buffers at temperatures above about 20°C, as evidenced by the lack of imino proton resonances in the region of 10-12 ppm in the NMR
- 10 spectrum of this sample (FIG. 9). In comparison, the corresponding natural sequence d(GGTTAGGGTTAG) forms G-quartet structures at temperatures as high as 70°C, as evidence by the imino proton resonances at 10-12 ppm observed in the NMR spectrum of this DNA (FIG. 8).

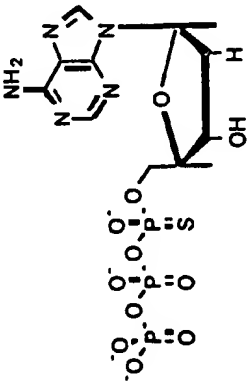
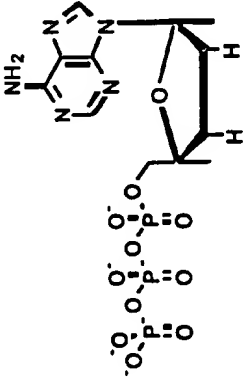
EXAMPLE 11

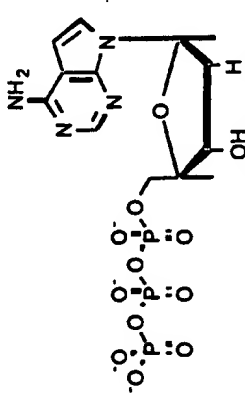
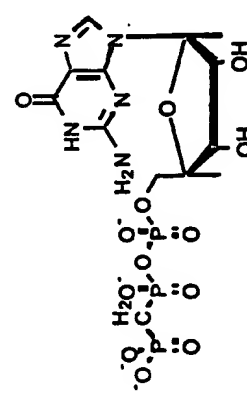
Table 12 summarizes results of various purine and pyrimidine analogs tested for activity as a telomerase inhibitor.

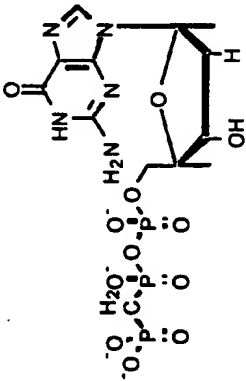
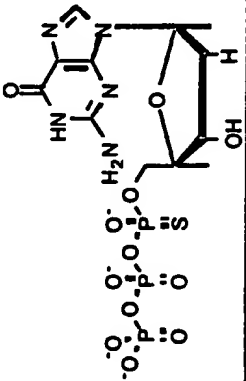
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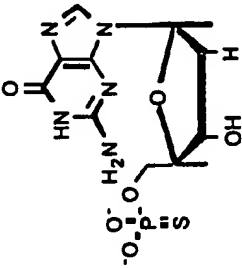
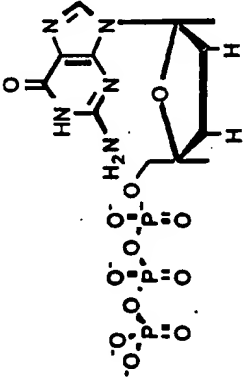
Inhibition of 293 Telomerase

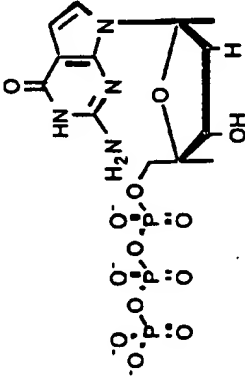
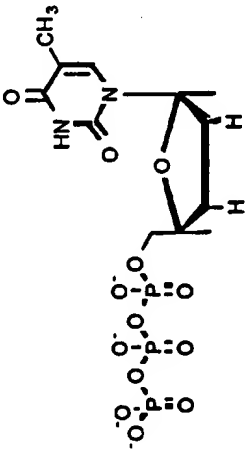
		Conventional Assay *dGTP	Modified Assay *dATP	Substrate of 293 Telomerase
NA004	2'-Deoxyadenosine-5'- β , γ -Methylene Triphosphate 	No Inhibition (Upto 2 mM)		Replace dATP Is a substrate No substrate inhibition

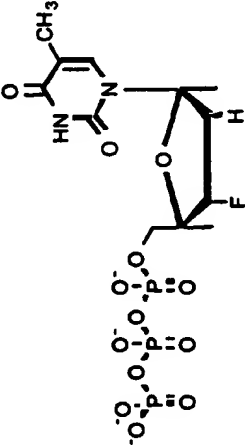
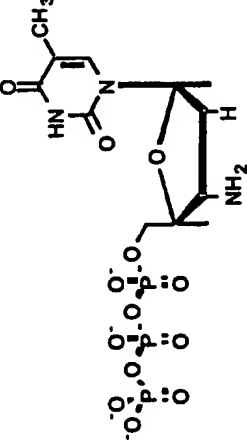
		Conventional Assay *dGTP	Modified Assay *dATP	Substrate of 293 Telomerase
NA006	<p>2'-Deoxyadenosine-5'-O-(1-Thio-Triphosphate)</p> 	No Inhibition (upto 2 mM)		Is a substrate No substrate inhibition
NA007	<p>2',3'-Dideoxyadenosine-5'-Triphosphate</p> 	No Inhibition (upto 2 mM)		No product formed using (TTAGGG) ₃ primer

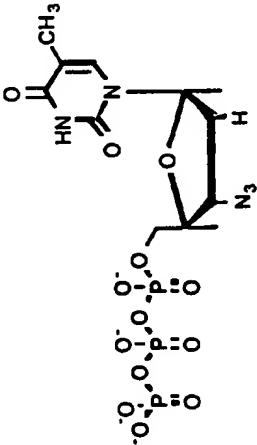
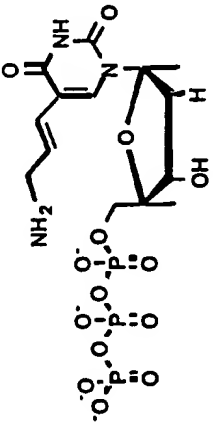
		Conventional Assay γ -dGTP	Modified Assay γ -dATP	Substrate of 293 Telomerase
NA02 3	7-Deaza-2'-Deoxyadenosine-5'-Triphosphate 	$IC_{50} = 59 \mu M$	Yes $IC_{50} = 8 \mu M$	Is a substrate Produces short telomere Substrate Inhibition
NA011	Guanosine-5'- β,γ -Methylene Triphosphate 	No Inhibition (upto 2 mM)		

		Conventional Assay *dGTP	Modified Assay *dATP	Substrate of 293 Telomerase
NA014	2'-Deoxyguanosine-5'-β,γ-Methylene Triphosphate 	(IC50 < 0.125 mM)		
NA013	2'-Deoxyguanosine-5'-O-(1-Thio-Triphosphate) 	(IC50 < 0.125 mM)		

		Conventional Assay *dGTP	Modified Assay *dATP	Substrate of 293 Telomerase
NA015	2'-Deoxyguanosine-5'-Phosphorothioate 	No Inhibition (upto 2 mM)		
NA020	2',3'-Dideoxyguanosine-5'-Triphosphate 	IC50<125 μM		

		Conventional Assay +dGTP	Modified Assay +dATP	Substrate of 293 Telomerase
NA02 2	<p>7-Deaza-2'-Deoxyguanosine-5'-Triphosphate</p> 	Yes (IC50 = 11 μM)	Yes (IC50 = 56 μM)	Is a substrate Produces short telomere Substrate Inhibition
NA001	<p>2',3'-Dideoxythymidine-5'-Triphosphate</p> 			

		Conventional Assay *dGTP	Modified Assay *dATP	Substrate of 293 Telomerase
NA002	3'-Fluoro-3'-Deoxythymidine-5'-Triphosphate 			
NA005	3'-Amino-3'-Deoxythymidine-5'-Triphosphate 			

		Conventional Assay *dGTP	Modified Assay *dATP	Substrate of 293 Telomerase
NA010	3'-Azido-3'-Deoxythymidine-5'-Triphosphate			
				
NA02 1	5-Allylamino-2'-Deoxyuridine-5'-Triphosphate	the telomerase activity is weak)		
				

5 All of the compositions and methods disclosed and claimed herein can be made
and executed without undue experimentation in light of the present disclosure. While the
compositions and methods of this invention have been described in terms of preferred
embodiments, it will be apparent to those of skill in the art that variations may be applied
to the composition, methods and in the steps or in the sequence of steps of the method
described herein without departing from the concept, spirit and scope of the invention.
More specifically, it will be apparent that certain agents which are both chemically and
physiologically related may be substituted for the agents described herein while the same
10 or similar results would be achieved. All such similar substitutes and modifications
apparent to those skilled in the art are deemed to be within the spirit, scope and concept
of the invention as defined by the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT: Chen, Shih-Fong
Maine, Ira
Kerwin, Sean M.
Fletcher, Terace
10 Salazar, Miguel
Mamiya, Blain
Wajima, Makoto
Windle, Bradford E.

15

(ii) TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR MODULATION
AND INHIBITION OF HUMAN TELOMERASE

(iii) NUMBER OF SEQUENCES: 4

20

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Arnold, White & Durkee
(B) STREET: P.O. Box 4433
(C) CITY: Houston
(D) STATE: Texas
25 (E) COUNTRY: United States of America
(F) ZIP: 77210

30

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

35

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US Unknown
(B) FILING DATE: Concurrently Herewith
(C) CLASSIFICATION: Unknown

40

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kitchell, Barbara S.
(B) REGISTRATION NUMBER: 33,928
(C) REFERENCE/DOCKET NUMBER: CTCR:028

45

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (512) 418-3000
(B) TELEFAX: (512) 474-7577

50

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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CAAACCCCA AAACC

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(2) INFORMATION FOR SEQ ID NO:3:

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- (B) TYPE: nucleic acid
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- (D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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18

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(2) INFORMATION FOR SEQ ID NO:4:

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

45

- (A) NAME/KEY: modified_base
 - (B) LOCATION: 7
 - (D) OTHER INFORMATION: /mod_base= OTHER
- /note= "N = G or 7-deaza-G"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

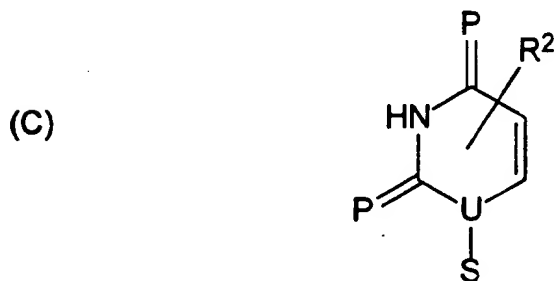
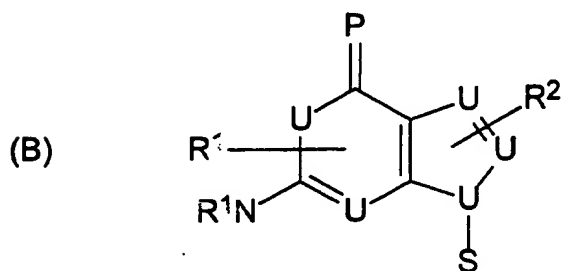
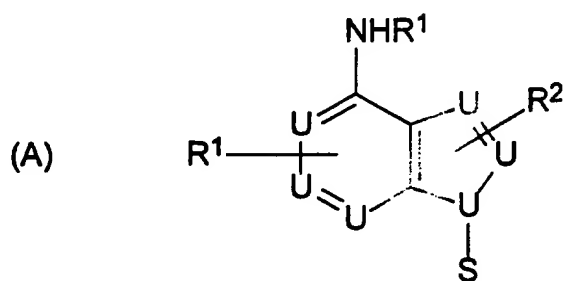
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12

WHAT IS CLAIMED IS:

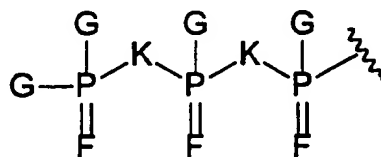
1. A method of reducing proliferative capacity of a cell comprising contacting said cell with a compound or a salt thereof or a stereoisomer of said compound that has the formula of (A), (B) or (C):



where U is independently carbon or nitrogen; R^1 is independently H, lower alkyl, or phenyl alkyl; R^2 is independently H, lower alkyl, NH_2 , halogen, azido or alkene; P is independently oxygen or sulfur; and S is an acyclic or cyclic glycosyl group represented by the formulae:

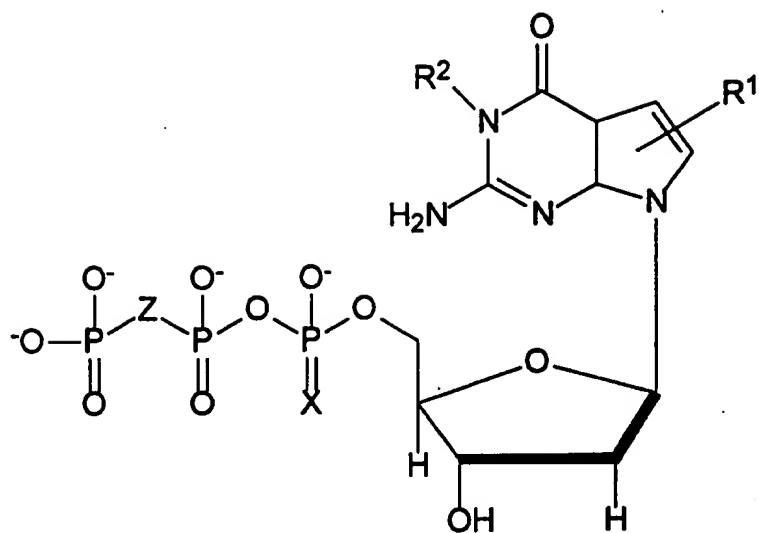


where R^3 is independently H, halogen, amino, azido, or hydroxyl; R^4 is independently H or OH; V is oxygen or methylene; and R^5 is OH, $(CH_2)_n$, $PO(OR^7)_2$, $OP(O)(OR^7)_2$; R^6 is H, lower alkyl, hydroxy-substituted lower alkyl, or haloalkyl; R^7 is H, lower alkyl, CH_2OCO (branched or straight chain alkyl (C1-C8) or aryl, and R^8 is H or CO (lower alkyl) and n is 1-2;



where K is oxygen or methylene; G is independently oxygen, sulfur or methyl; F is independently oxygen or sulfur; and the glycosyl bond between S and U is α or β ;

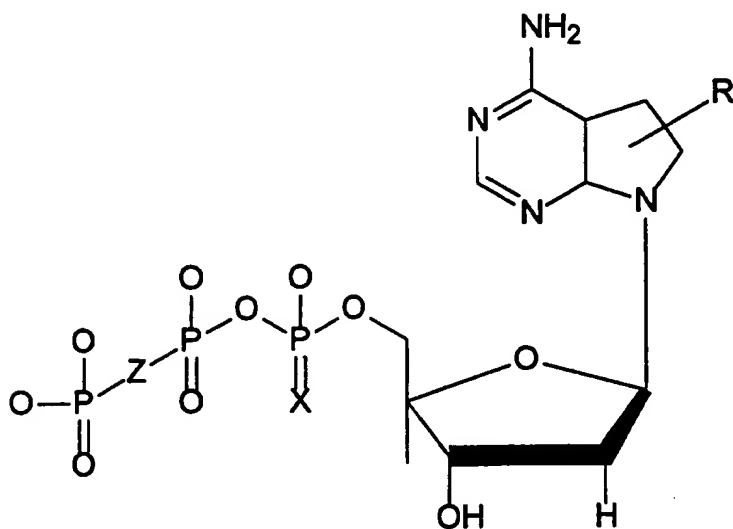
2. The method of claim 1 wherein the compound is further identified as having the formula or the corresponding nucleoside thereof:



where R is lower alkyl or halogen; Z is oxygen or methylene; and X is sulfur or oxygen.

5

3. The method of claim 1 wherein the compound is further identified as having the formula:



where R¹ is H, lower alkyl, or halogen; R² is H or lower alkyl; Z is oxygen or methylene and X is sulfur or oxygen or the corresponding nucleoside thereof.

5

4. The method of claim 1 wherein the compound is further identified as 2'-deoxyadenosine-5'-β,γ-methylene triphosphate, 2'-deoxyadenosine-5'-O-(1-thio) triphosphate, 2'-deoxy guanosine-5'-O-(1-thio) triphosphate, 7-deaza-2'-deoxy guanosine-5'-β,γ-methylene triphosphate, 7-deaza-2'-deoxyguanosine-5'-triphosphate or 7-deaza-2'-deoxyadenosine-5'-triphosphate or the corresponding nucleoside thereof.

10

5. The method of claim 1 wherein the compound is identified as 2,3'-dideoxythymidine-5'-triphosphate, 3'-azido-3'-deoxythymidine-5'-triphosphate, 3'-amino-3'-deoxythymidine-5'-triphosphate, 5-allylamino-2'-deoxyuridine-5'-triphosphate, guanosine-5'-β,γ-methylene triphosphate, 2',3'-dideoxyguanosine-5'-triphosphate, 2',3'-dideoxyadenosine-5'-triphosphate or the corresponding nucleoside thereof.

15

20

6. The method of claim 1 where the cell is in a mammal.

7. The method of claim 1 wherein the cell is a human cell.

25

8. The method of claim 1 wherein the cell is further identified as a cancer cell.

9. A method of prematurely shortening a telomere comprising

administering an amount of 7-deaza nucleoside in an amount sufficient to inhibit telomere extension.

10. The method of claim 1 wherein the reduced proliferative capacity leads to cell
5 death.

11. The method of claim 9 wherein the nucleoside is identified as having the following structure:

10

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12. The method of claim 1 wherein the nucleoside is 7-deaza dGDP.

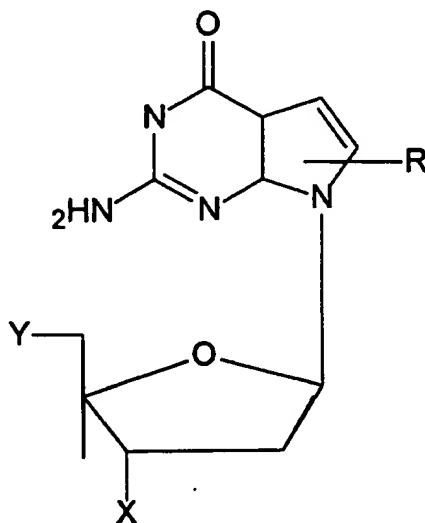
20 13. The method of claim 1 wherein the nucleoside is 7-deaza dADP.

14. A method of converting processive telomerase to non-processive telomerase comprising providing a telomerase-containing cell with an amount of a deaza purine
25 nucleoside sufficient to incorporate into telomeric DNA or by cleaving telomerase RNA component.

15. The method of claim 13 wherein the purine nucleotide is further identified as 2'-deoxyadenosine-5'- β,γ -methylene triphosphate, 2'-deoxyadenosine-5'-O-(1-thio) triphosphate, 2'-deoxy guanosine-5'-O-(1-thio) triphosphate, 7-deaza-2'-deoxy guanosine-5'- β,γ -methylene triphosphate, 7-deaza-2'-deoxyguanosine-5'-triphosphate, 7-deaza-2'-deoxyadenosine-5'-triphosphate or 2'3'-dideoxyadenosine-5'-triphosphate.

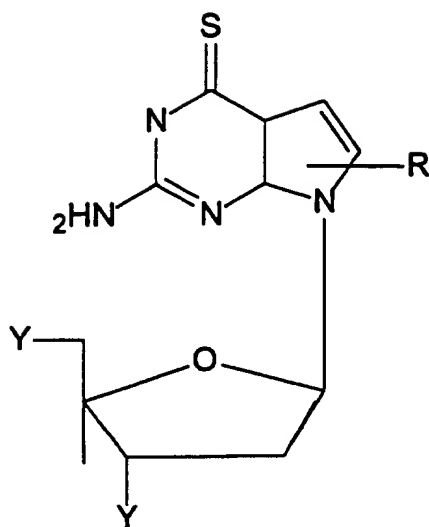
16. A method of inhibiting G-quartet formation in a telomere, comprising providing a telomerase-containing cell with an amount of a 7-deaza-2'-deoxyguanosine sufficient to incorporate into telomeric DNA in the presence of the telomerase.

17. The method of claim 15 wherein the 7-deaza-2'-deoxyguanosine is further identified as having the formula:



where R is H, C1-C5 alkyl or phenylalkyl; X is H or OH; and Y is OH or 1-thio-triphosphate or triphosphate.

18. A compound of the formula:



5

where R^1 is independently H or lower alkyl; R^2 is H, halogen, or lower alkyl; and Y is independently OH or OTol where Tol is toluoyl or a pharmaceutically acceptable composition or stereochemical isomer thereof.

10 Exceptions for known compounds:

except when $Y=OH$, $R^2=H$ then E^1 cannot equal H

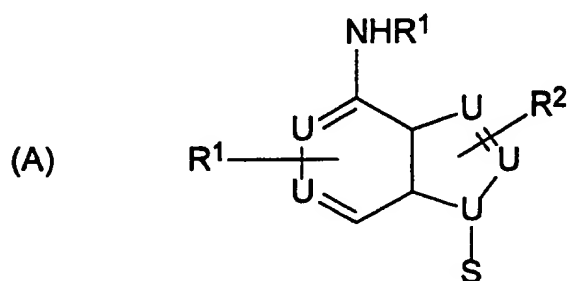
: when $Y=OTol$, $R^2=H$ then E^1 cannot equal H

15

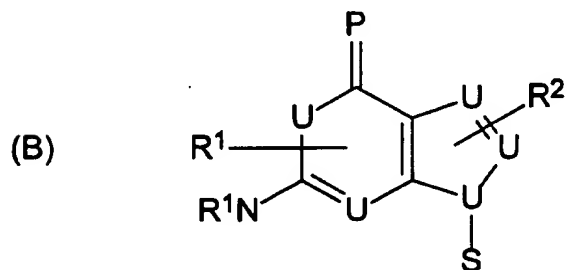
19. The compound of claim 12 which is 1-methyl-2-amino-7-[2'-deoxy-3-5-di-O-(p-toluoyl)- β -D-erythropentofuranose]7H-pyrrolo[2,3-d]pyrimidine-4-(3H)-thione.

20. The compound of claim 12 which is 1-methyl-2-amino-7-[2'deoxy- β -D-erythropentofuranose]-7H-pyrrolo[2,3-d]pyrimidine-4-(3H)-thione.

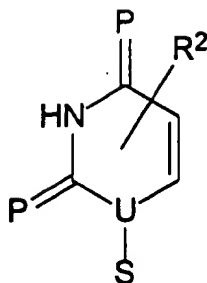
- 5 21. A method of producing chromosome instability comprising treating a chromosome containing cell with a composition containing a compound having the formula:



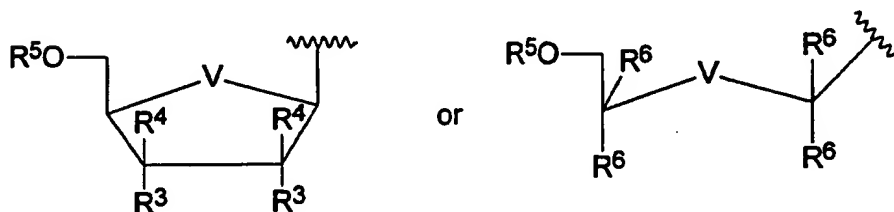
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(C)



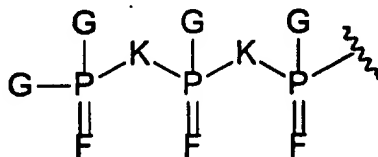
- 5 where U is independently carbon or nitrogen; R^1 is independently H, lower alkyl, or phenyl alkyl; R^2 is independently H, lower alkyl, NH_2 , halogen, azido or alkene; P is independently oxygen or sulfur; and S is an acyclic or cyclic glycosyl group represented by the formulae:



10

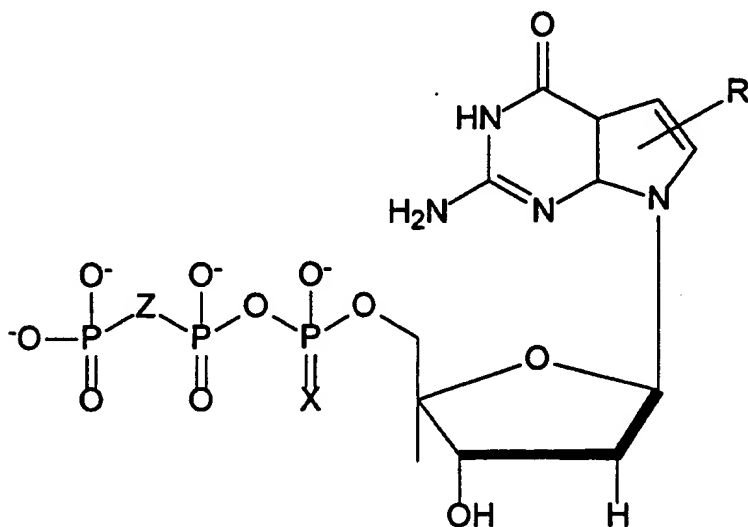
- where R^3 is independently H, halogen, amino, azido, methylene or hydroxyl; R^4 is independently H or OH; V is oxygen or carbon; and R^5 is OH, $(CH_2)_n$, $PO(OR^7)_2$, $OP(O)OR^7$; R^6 is H, lower alkyl, hydroxy-substituted lower alkyl, or haloalkyl; R^7 is H, lower alkyl, CH_2O_2C (lower alkyl) or aryl, and R^8 is H or CO(lower alkyl);

15



where K is oxygen or carbon; G is independently oxygen, sulfur or methyl; F is independently oxygen or sulfur; n is 1-2; and the glycosyl bond between S and U is α or β ;

- 5 22. The method of claim 1 wherein the compound is further identified as having the formula or the corresponding nucleoside thereof:



where R is lower alkyl or halogen; Z is oxygen or methylene; and X is sulfur or oxygen.

10

23. The method of claim 20 wherein the chromosome instability is by interference with DNA repair process.

15

24. The method of claim 20 wherein the chromosome instability is by inducing telomeric DNA instability.

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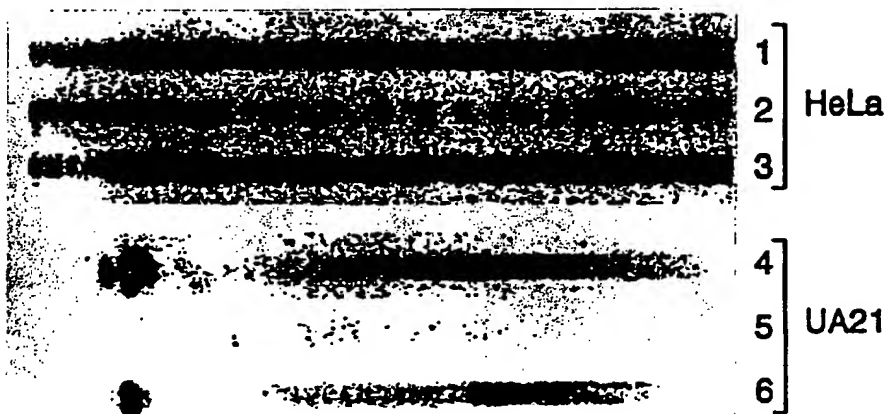


FIG.1

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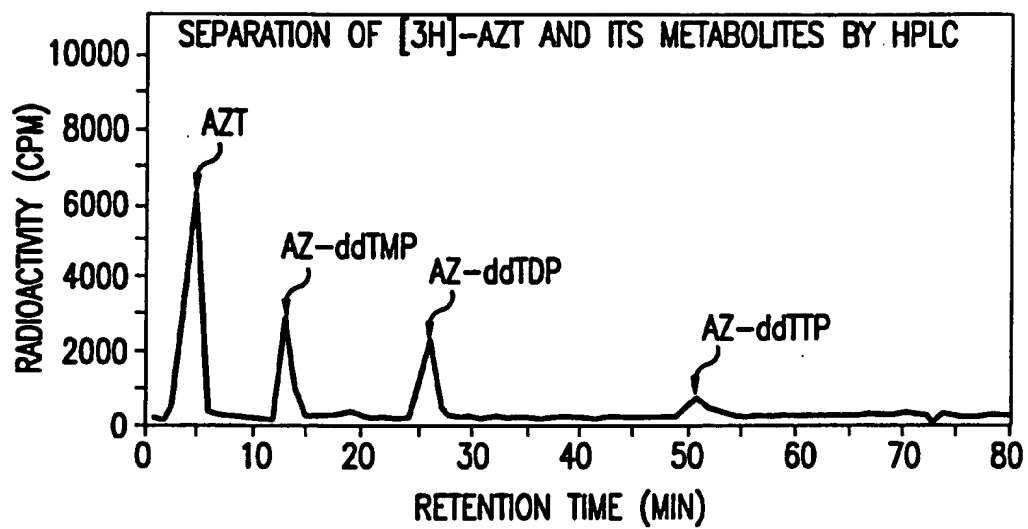


FIG.2

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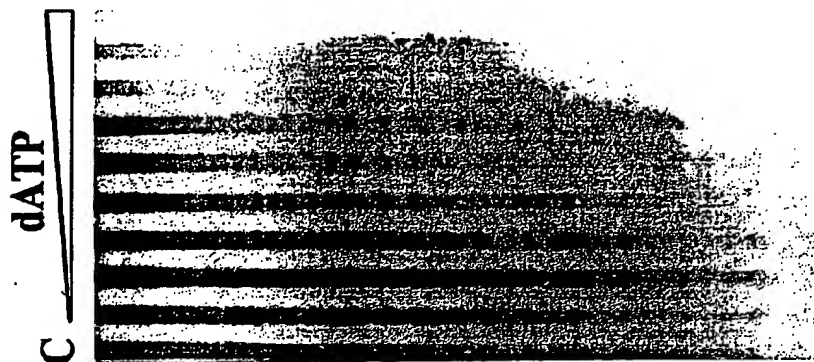


FIG.3D

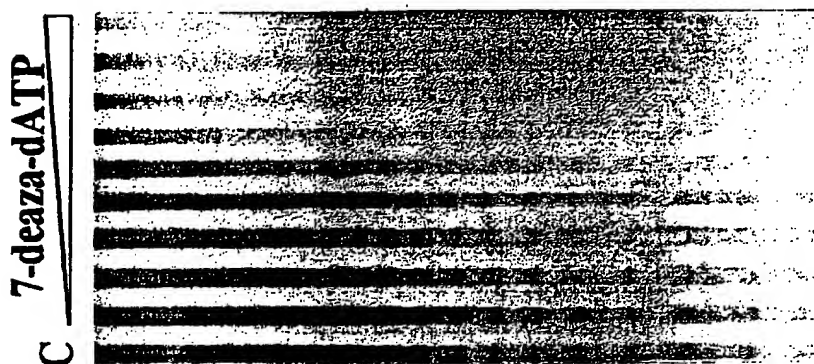


FIG.3C

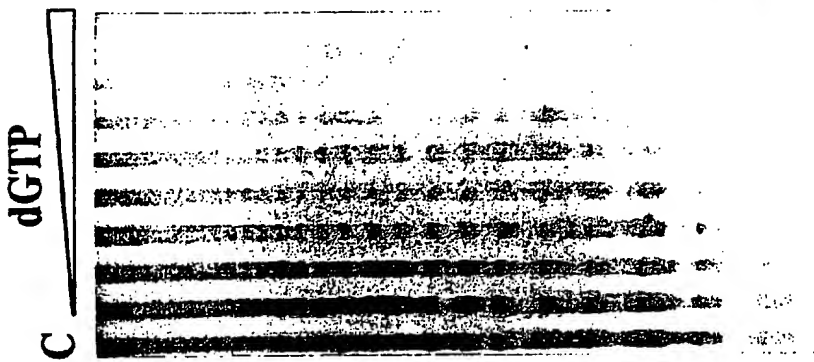


FIG.3B

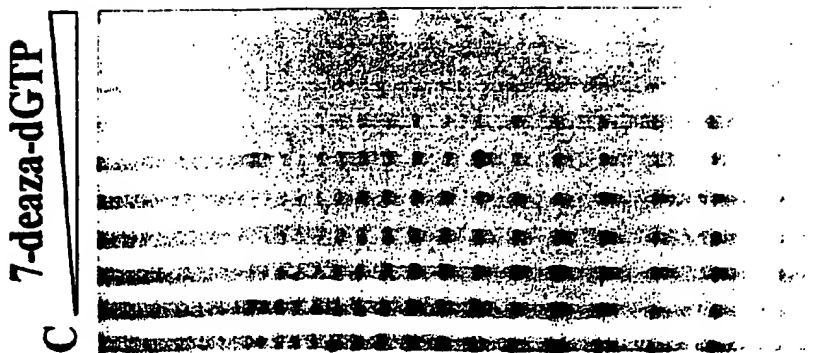


FIG.3A

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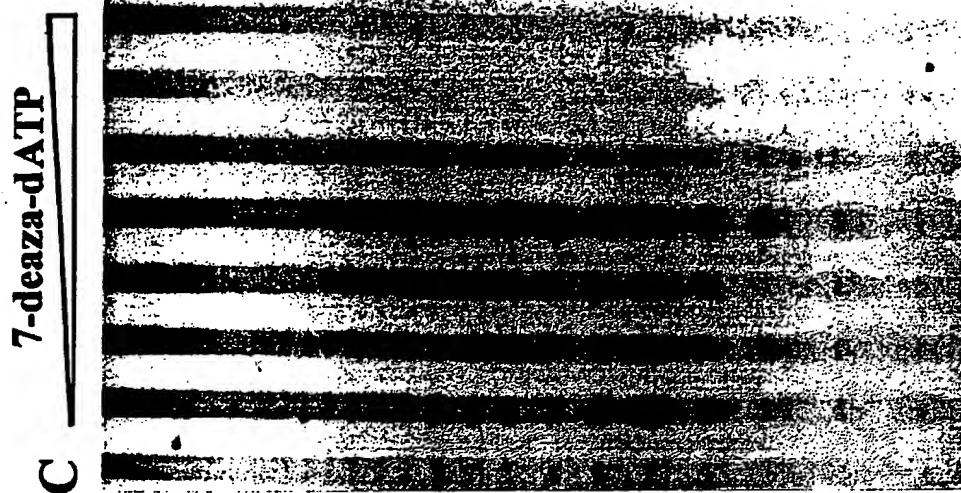


FIG.4B

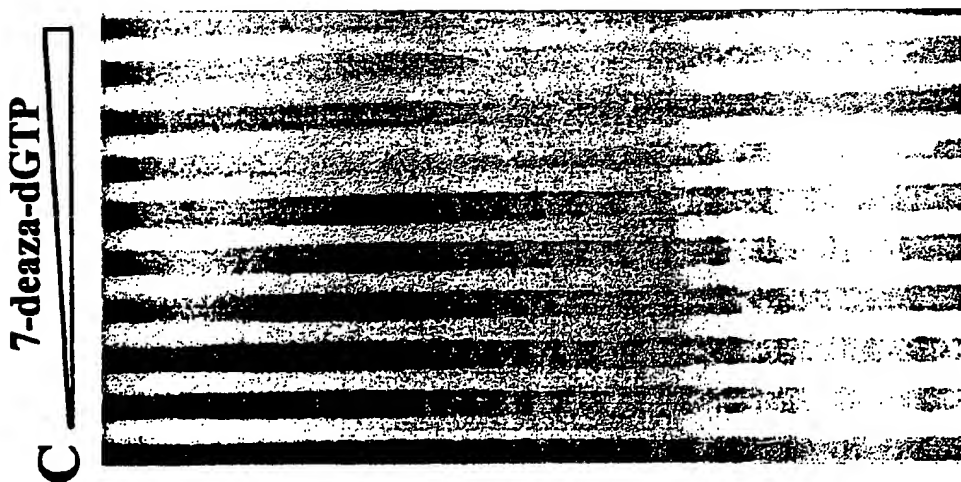


FIG.4A

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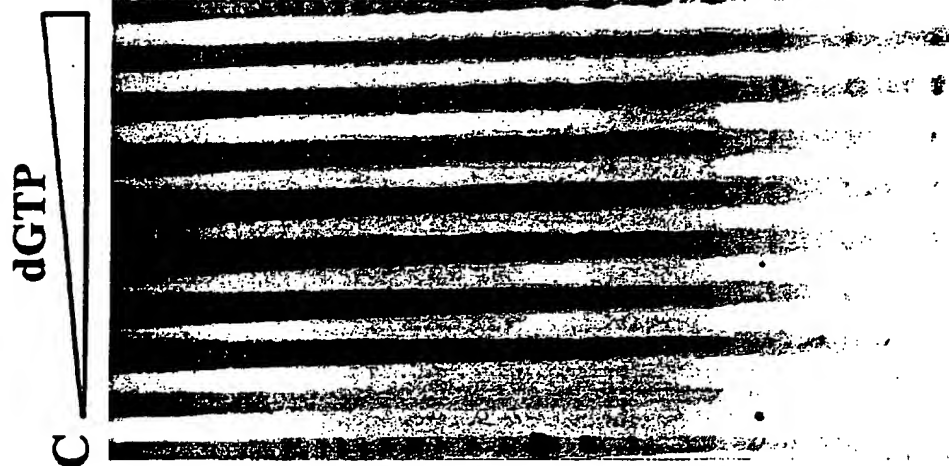


FIG. 5C

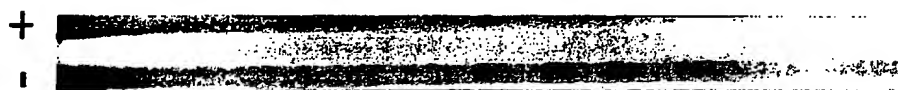


FIG. 5B

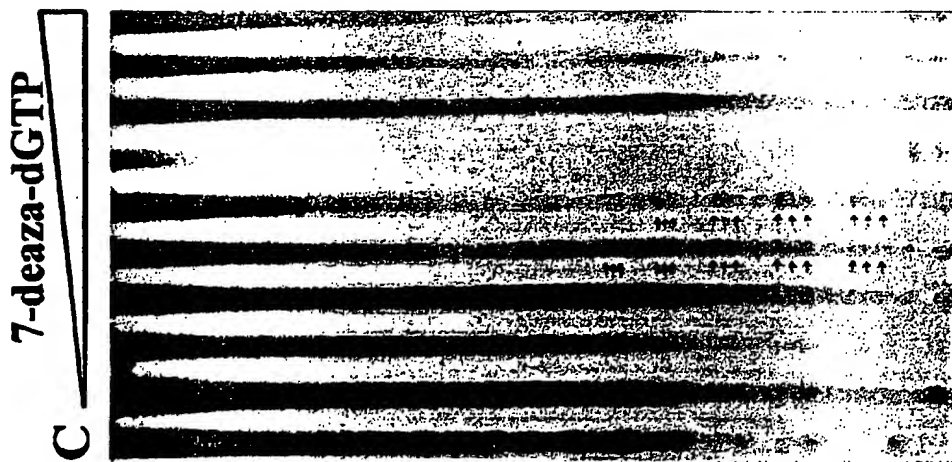


FIG. 5A

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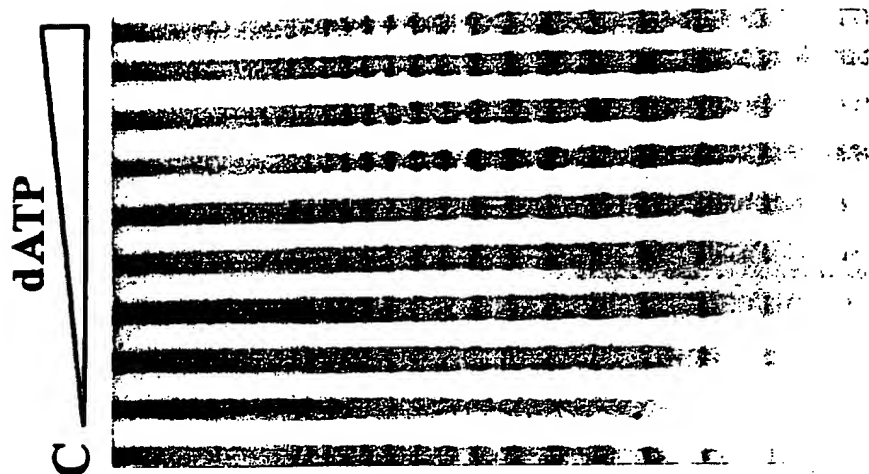


FIG. 6C

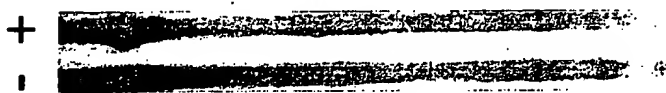


FIG. 6B

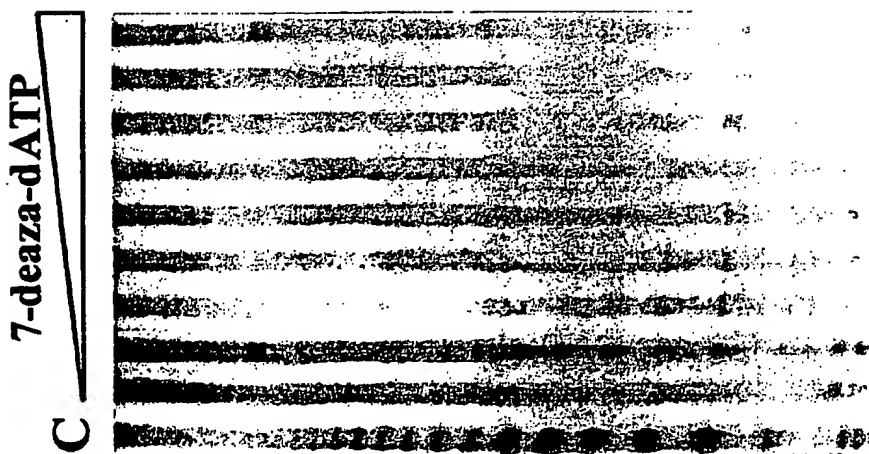


FIG. 6A

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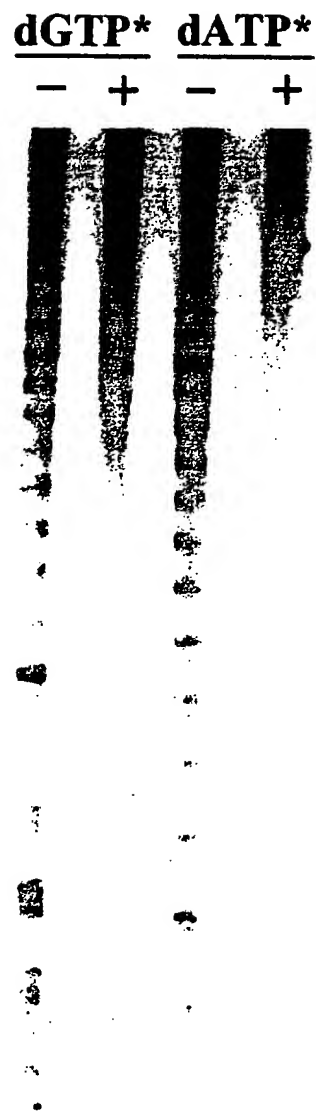
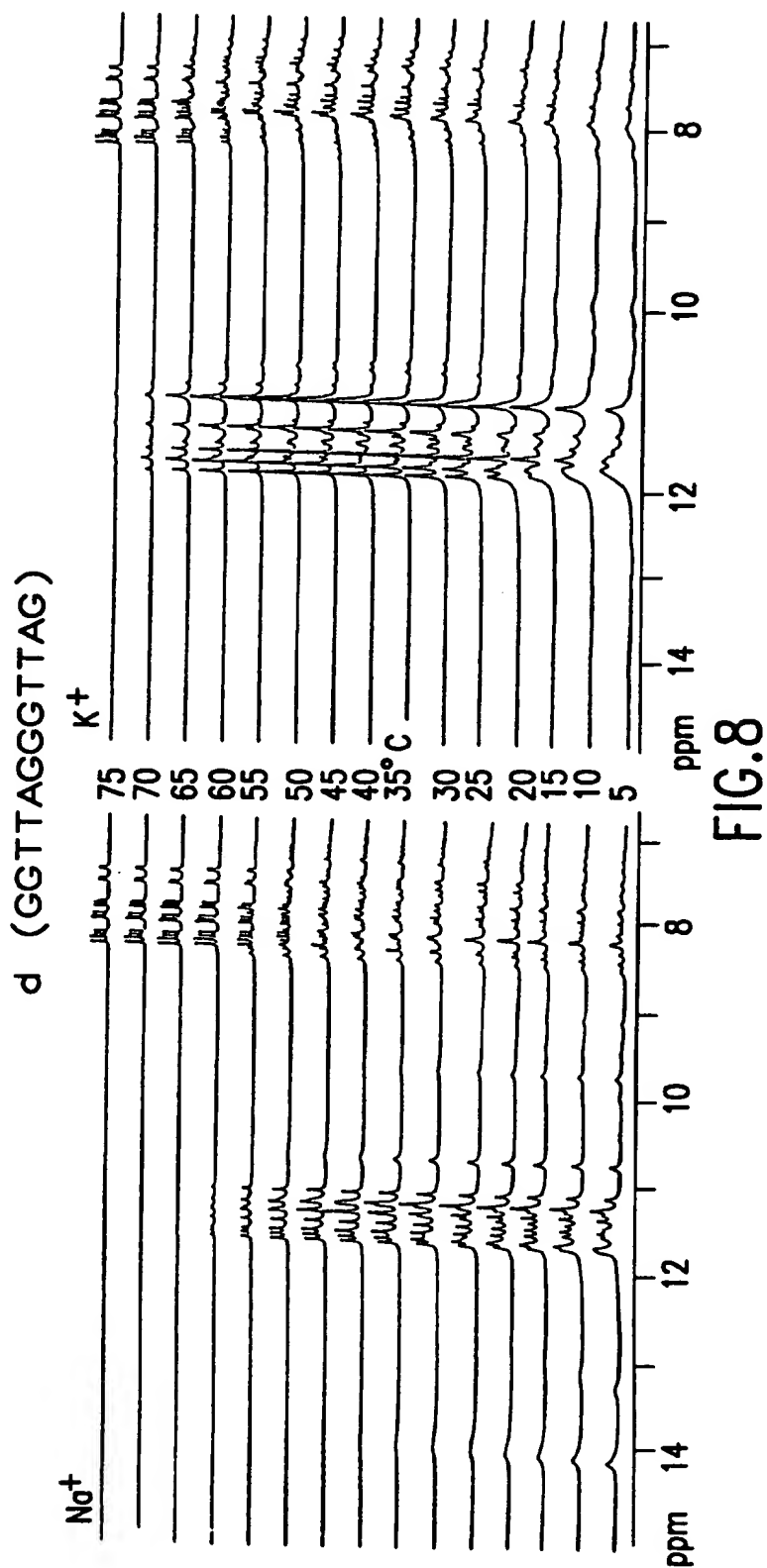


FIG. 7

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SUBSTITUTE SHEET (RULE 26)

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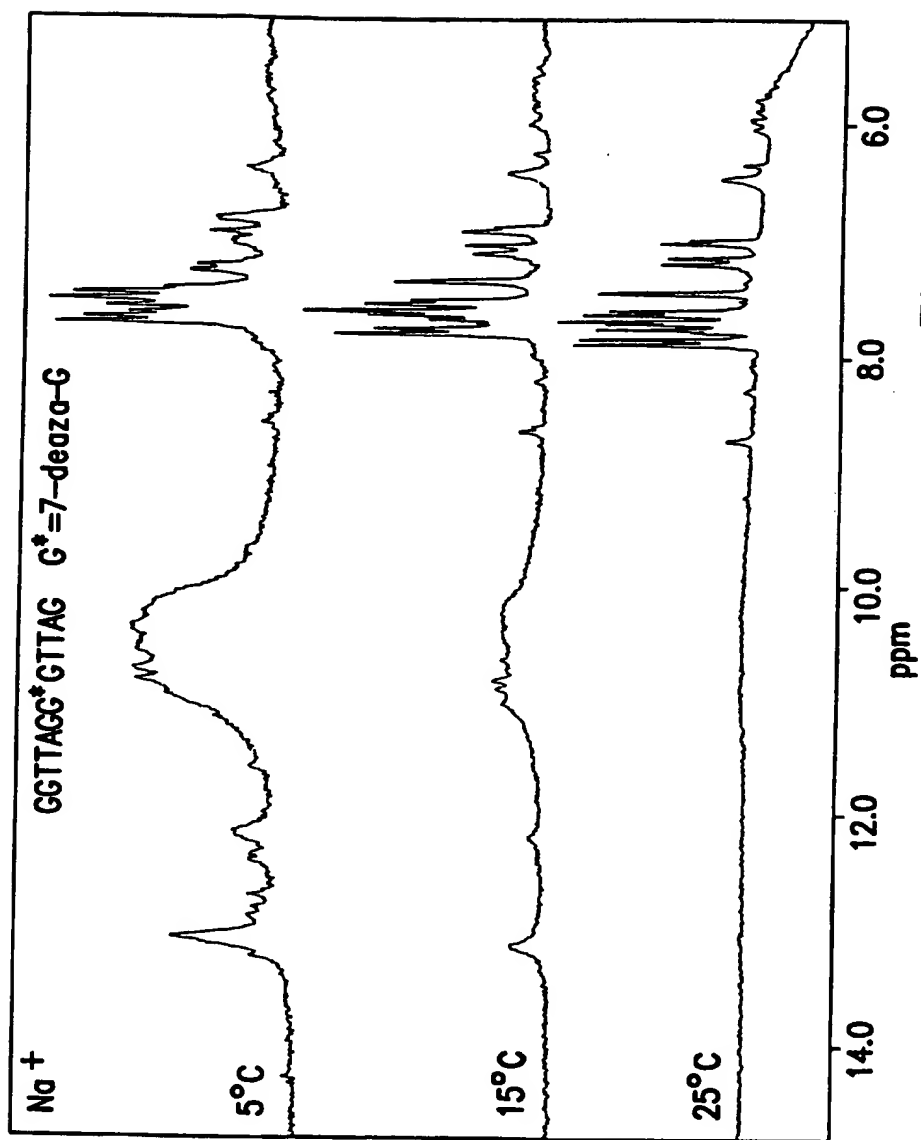


FIG.9

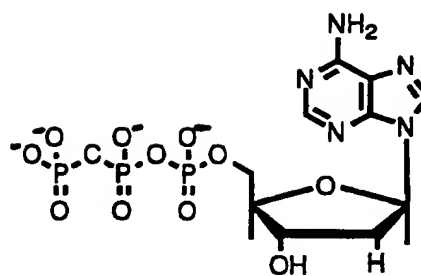
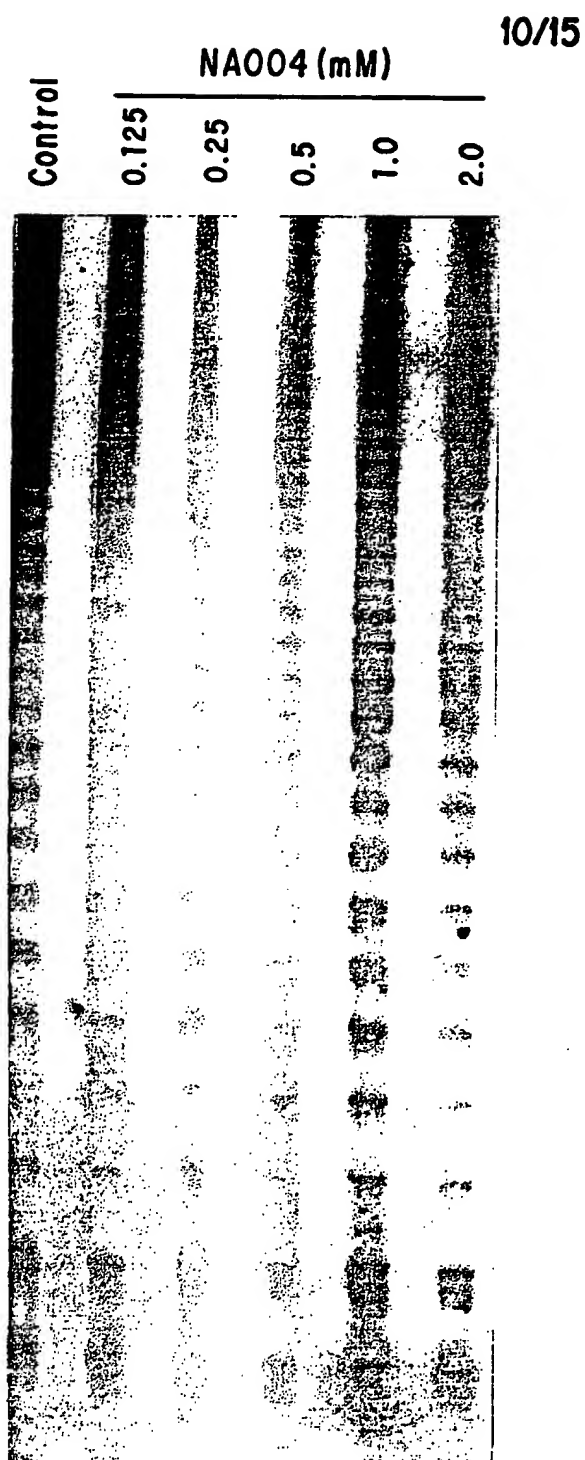


FIG.10

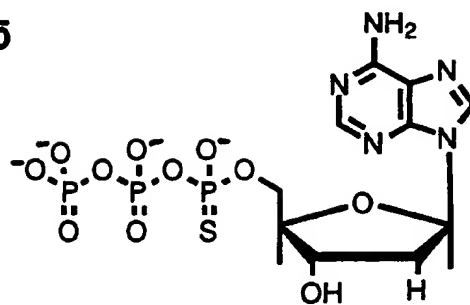
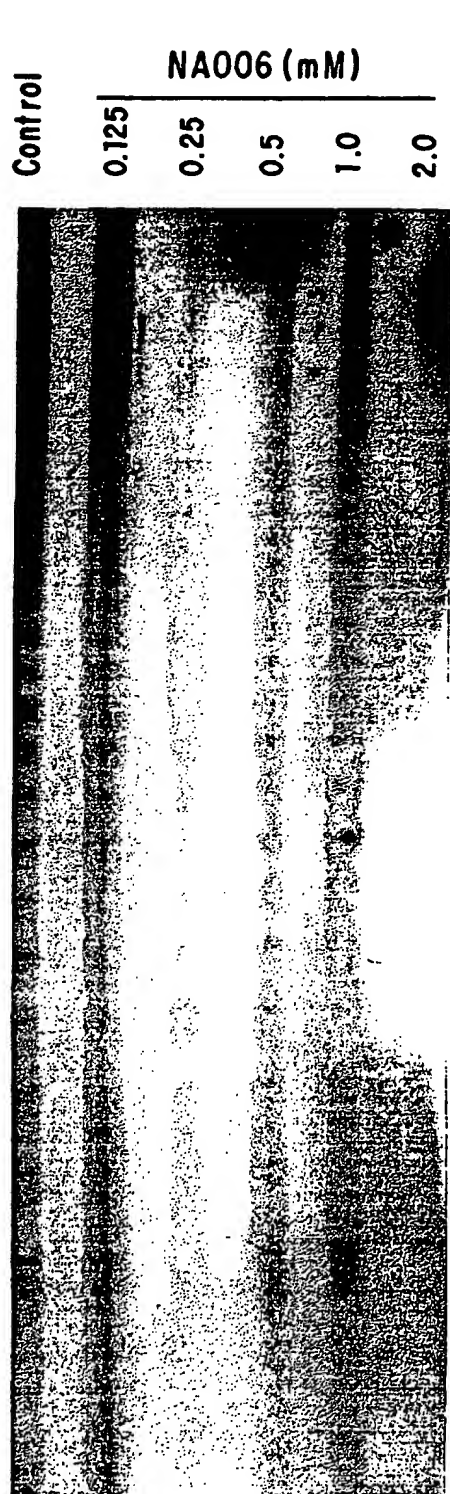


FIG.11

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Control	NA007 (mM)				
	0.125	0.25	0.5	1.0	2.0

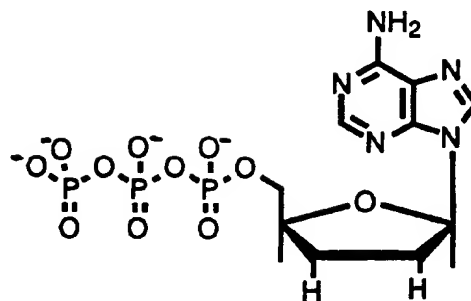
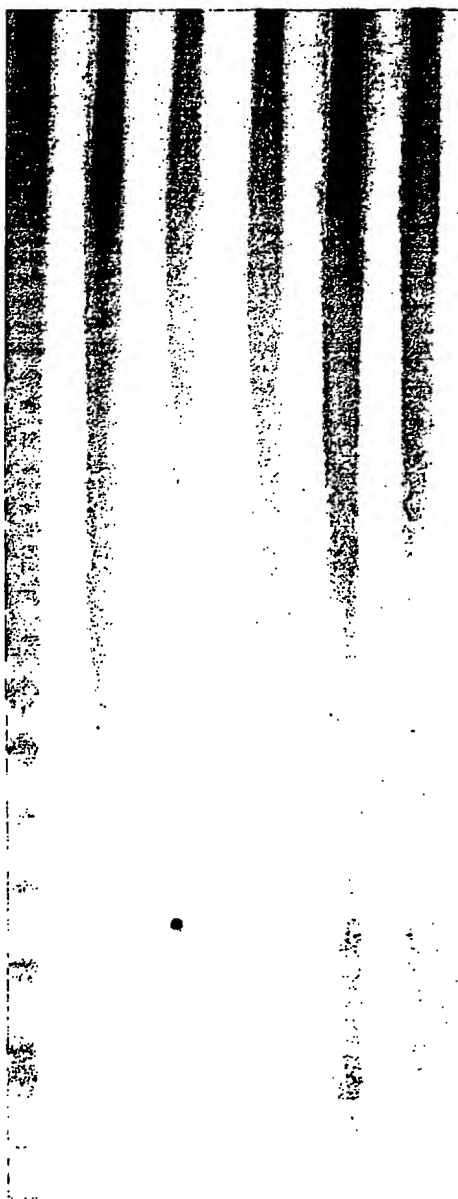


FIG.12

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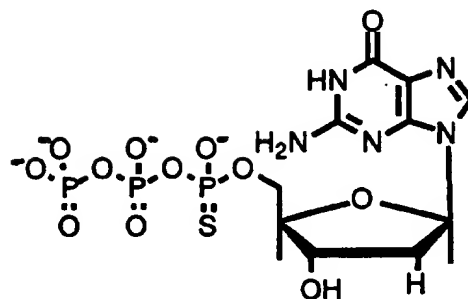


FIG. 13

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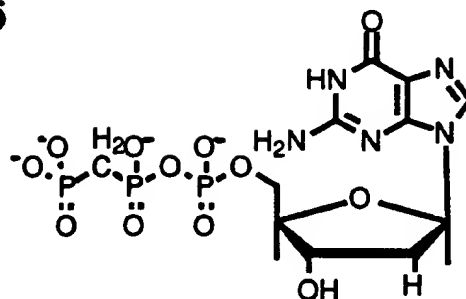
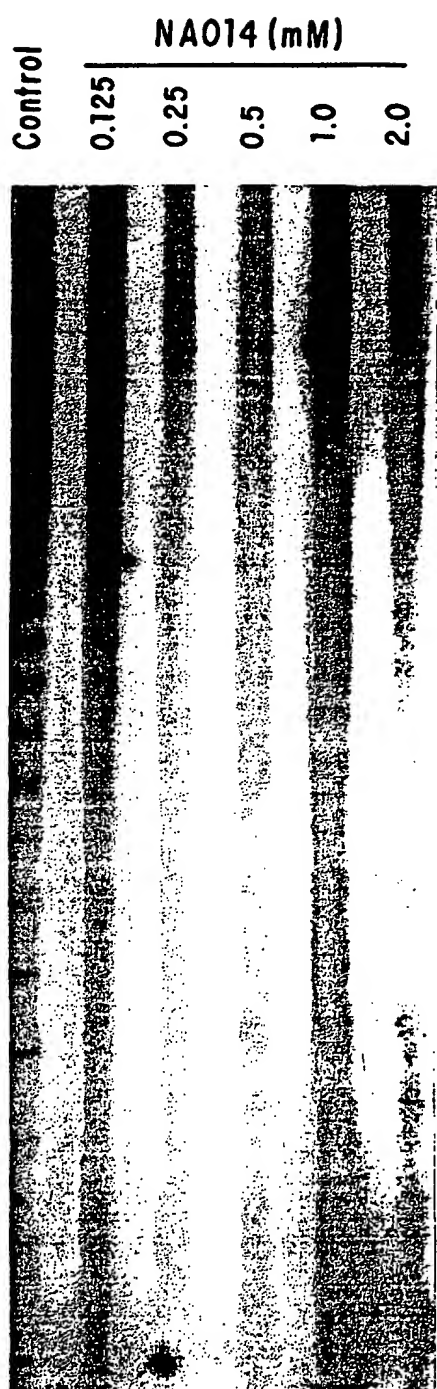
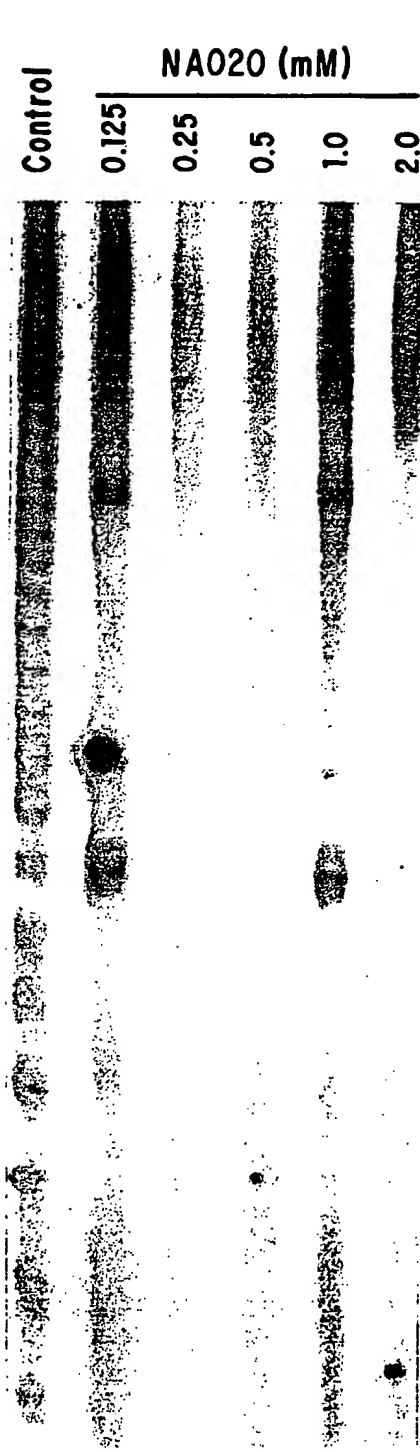


FIG. 14



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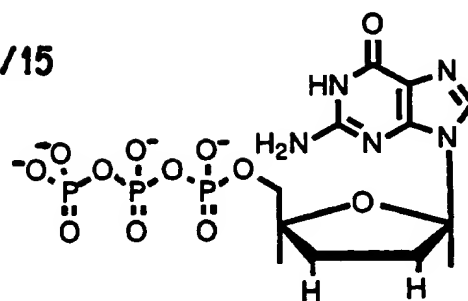


FIG.15

INTERNATIONAL SEARCH REPORT

International application No

PCT/US 96/11421

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07H19/02 C07H19/10 C07H19/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEIC ACIDS RES., vol. 22, 1994, pages 893-900, XP002019881 E.H. BLACKBURN AND C. STRAHL: "The effects of nucleoside analogs on telomerase and telomeres in Tetrahymena" cited in the application see the whole document	1,4-8, 10,15,21
P,X	MOL. CELL. BIOL., vol. 16, no. 1, 1996, pages 53-65, XP002019882 E.H. BLACKBURN AND C. STRAHL: "Effects of Reverse Transcriptase Inhibitors on Telomere Length and Telomerase Activity in Two Immortalized Human Cell Lines" cited in the application see the whole document	1,4-8, 10,15,21

-/-



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "A" document member of the same patent family

Date of the actual completion of the international search

2 December 1996

Date of mailing of the international search report

03.01.97

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Authorized officer

Bardili, W

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/11421

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. BIOL. CHEM., vol. 34, 1994, pages 21858-69, XP002019883 S.K. BRAHMACHARI ET AL.: "Structure and Stability of Human Telomeric Sequence" cited in the application see the whole document ---	16
A	SCIENCE, vol. 266, 1994, pages 2011-4, XP002019884 N.W. KIM ET AL.: "Specific Association of Human Telomerase Activity with Immortal Cells and Cancer" cited in the application see the whole document ---	1-10, 12-24
A	J. MED. CHEM., vol. 33, 1990, pages 1220-5, XP002020030 R. REVANKAR ET AL.: "Synthesis and Antitumor Evaluation in Mice of Certain 7-Deazapurine (Pyrrolo(2,3-d)pyrimidine) and 3-Deazapurine (Imidazo(4,5-c)pyridine) Nucleosides" see 'scheme I' ---	18
X	CHEMICAL ABSTRACTS, vol. 119, no. 1, 1993 Columbus, Ohio, US; abstract no. 123, "Anticancer Activities of Adenine Nucleotides in Tumor Bearing Hosts" page 7; XP002020031 see abstract & DRUG DEV. RES., vol. 28, 1993, pages 428-31, ---	1
X	CHEMICAL ABSTRACTS, vol. 100, no. 23, 1984 Columbus, Ohio, US; abstract no. 185159, R.K. ROBINS: "The potential of nucleotide anlogs as inhibitors of retroviruses and tumors" page 3; XP002020032 see abstract & PHARM. RES., vol. 1, 1984, pages 11-8, ---	1

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/11421

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 81, no. 9, 1974 Columbus, Ohio, US; abstract no. 45178, C.T. HARDESTY ET AL.: "Disposition of the Antitumor Agent Sangivamycin" XP002020033 see abstract & CANCER RES., vol. 34, 1974, pages 1005-9,	1
X	--- CHEMICAL ABSTRACTS, vol. 101, no. 19, 1984 Columbus, Ohio, US; abstract no. 163078, P.F. TORRENCE: "The chemistry and biochemistry of purine and pyrimidine nucleoside antiviral and antitumor agents" page 3; XP002020034 see abstract & DRUGS PHARM. SCI., vol. 24, 1984, pages 113-76, -----	1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JS 96/11421

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 11
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Claimed structure is missing
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210.

Incomplete Search US96/11421:

The present application contains essential errors in the claims and in the corresponding parts of the description:

Claims 1, 21: Although the triphosphate residue represented by a formula in the claim has a free valence, its attachment to the preceding structure in the claim is not defined. For the purpose of the Search, it has assumed that the triphosphate residue is attached to a 5'-hydroxy group of the sugar part (S).

Also, radical R^a , defined as H or CO(lower alkyl), is not part of any of the formulae depicted in the claim. Its meaning is obscure.

Claim 2: Meaning of radical R not defined. One central double bond in the purine ring is missing. This becomes immediately evident when the claim is compared with the examples set forth in the specification. For the purpose of the Search, the bond has been taken into account.

Claim 3: Meaning of radicals R^1 and R^2 not defined. For the purpose of the Search, it has been assumed that the definitions in claim 2 and 3 have been exchanged erroneously. Also, two double bonds in the purine ring are missing. For the purpose of the Search, the bonds have been taken into account.

Claim 11: Claimed structure is missing.

Claim 17: Missing substituent on N-3 of the purine ring. Also, one central double bond in the purine ring is missing. For the purpose of the Search, the bond has been taken into account.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

-2-

Claim 18: Radicals R^1 and R^2 not present in the formula. It is not clear whether R^1 is present at the free N-3 or at the 5-membered ring. The same applies to R^2 . For the purpose of the Search, it has been assumed that R^1 is attached to N-3 and R^2 is attached to the 5-membered ring. Also, one central double bond in the purine ring is missing. For the purpose of the Search, the bond has been taken into account.

Also, in the disclaimer E^1 should be R^1 .

Claims 21, 22: One central double bond in the purine ring is missing in (A). For the purpose of the Search, the bond has been taken into account.

Consequently, claim 11 could not be searched. All other claims have been fully searched on the basis of the above interpretation of the claims.